Analysis of Mechanisms Regulating Prolactin Gene Expression

By

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CERTIFICATE OF APPROVAL

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ABSTRACT

Prolactin is a pleiotropic anterior pituitary peptide hormone. Perhaps the best known activity involves stimulation of lactation in mammals. Expression of the prolactin gene in the anterior pituitary is cell-type specific and regulated by hypothalamic and other hormones. Although the prolactin gene has been extensively studied, there remain substantial gaps in the understanding of the mechanisms mediating regulated, cell-specific expression of this physiologically important gene. One part of this thesis focuses on analysis of the role of specific transcriptional activator proteins in mediating basal and hormone-regulated expression of the prolactin promoter. The other will explore the events mediating cAMP-induced activation of the prolactin promoter.

The first part of the thesis explores the role of the related transcriptional co-activator proteins CREB binding protein (CBP) and p300 in the activation of the prolactin promoter. Expression vectors for p300 were found to enhance the synergistic, Pit-1/Ets-1 dependent activation of the prolactin promoter. In vitro binding studies demonstrated that p300 binds to a prolactin promoter DNA element in a Pit-1 and/or Ets-1 dependent manner. Mutational analysis of p300 provided evidence that no one particular structural or functional domain was responsible for the Pit-1/Ets-1 dependent enhancement of the prolactin promoter activity. However, the modest ability of p300 to activate the prolactin promoter limits interpretation of these studies. To determine if CBP and/or p300 are required to mediate transcriptional activation by Pit-1 and/or Ets-1, an expression

vector for the adenovirus 12S E1A protein, which blocks CBP/p300 activity, was used. The results demonstrate that E1A greatly reduced the ability of Pit-1, Ets-1 or the combination of the two factors to activate the prolactin promoter in heterologous, COS cells. E1A completely blocked activation of the prolactin promoter by the EGF/Ras pathway while only partially blocking activation by the forskolin/PKA pathway in GH3 cells suggesting the existence of a p300-independent activation component of the forskolin/PKA pathway

The second part of this thesis analyzes the possible role for the mitogen activated protein kinase (MAPK) in mediating cAMP activation of the prolactin promoter. These studies have used GH_3 pituitary tumor cells which express the prolactin gene. The results demonstrate that elevation of cAMP levels in GH_3 cells leads to activation of MAPK, consistent with a possible role for MAPK in mediating responses to cAMP. Inhibition of MAPK activation by the specific inhibitor, PD98059, was found to reduce the ability of cAMP to stimulate prolactin promoter activity. Thus, MAPK appears to be required for full responsiveness of the prolactin promoter to cAMP. Previous studies of other signaling pathways have provided evidence that Ets sites in the prolactin promoter probably play a role in mediating responsiveness to MAPK activation. In view of the ability of cAMP to activate MAPK in GH_3 cells, it seemed likely that Ets sites would also play a role in cAMP-responsiveness. Surprisingly, disruption of all known Ets sites within the proximal region of the prolactin promoter did not abrogate responsiveness to cAMP. Thus, while many signaling pathways act through

MAPK, surprisingly, not all appear to require Ets sites for the activation of the prolactin gene.

ABBREVIATIONS

ACTH..... ... adrenocorticotropin hormone

ADH..... anti-diuretic hormone

βgal..... ..... beta galactosidase

BRD..... bromodomain

CBP.....CREB binding protein

CMV..... cytomegalovirus

CPT-cAMP..... chloro-phenyl-thio-cyclic-AMP

CREB..... cAMP response element binding protein

DMEM..... Dulbecco's modified eagle's medium

EGF..... epidermal growth factor

ERK..... extracellular regulated kinase

FSH..... follicle stimulating hormone

FSK..... forskolin

GH..... growth hormone

GST..... glutathione-s-transferase

HAT..... histone acetyltransferase

HRP..... horse radish peroxidase

LH..... lutenizing hormone

Lhx3..... lim homeodomain transcription factor 3

MAPK..... mitogen activated protein kinase

NR..... nuclear hormone receptor

P/CAF..... p300/CBP associated factor

PCR..... polymerase chain reaction

PKA..... protein kinase A

PKI.....protein kinase A inhibitor

PRL..... prolactin

RSV..... rous sarcoma virus

TBP..... TATA binding protein

TBS..... ..... tris buffered saline

TFIIB..... transcription factor IIB

TRH..... thyrotropin releasing hormone

TSH..... thyroid stimulating hormone

CHAPTER 1

INTRODUCTION

Prolactin, an anterior pituitary peptide hormone, is an important regulator of mammalian growth, development, and homeostasis. Expression of the prolactin gene in the anterior pituitary is cell type specific and tightly regulated by several hormones and growth factors. Various disease states ranging from abnormal reproductive events to certain types of cancer have been associated with unregulated prolactin serum levels. The molecular mechanisms of regulated prolactin gene expression have not been completely elucidated. This thesis examines the specific interaction between three transactivating factors thought to be important for the regulated expression prolactin; Pit-1, Ets-1, and CBP/p300 and explores the mechanisms involved in cAMP induced regulation of the prolactin gene . The following introduction section will begin with a discussion of basic mammalian anterior pituitary anatomy, then continue on to discuss prolactin itself, prolactin associated diseases, and the regulation of the prolactin gene by various hormones and growth factors. This will be followed by a more in depth discussion of the prolactin promoter and transactivating factors thought to be involved in the regulation of prolactin expression. Finally, the known, pair-wise interactions of Pit-1, Ets-1, and/or CBP/p300 and their involvement in the activation of expression of specific genes will be discussed.

The Mammalian Pituitary

The mammalian pituitary gland plays a critical role in the regulation of growth, development, and homeostasis. The pituitary is comprised of separate anterior and

posterior lobes. Each lobe is directly influenced by hormones released from the hypothalamus. However, the physical connection between the hypothalamus and each pituitary region is anatomically distinct (Fig.1). The posterior pituitary is essentially an outgrowth of the hypothalamus and as such is comprised of neural tissue. The two hormones released by the posterior pituitary, oxytocin and vasopressin or ADH, are actually synthesized within the cell bodies of hypothalamic neurons. The hormones are transported down the axons of the hypothalamic neurons to capillary beds within the posterior pituitary where they are released into general circulation.

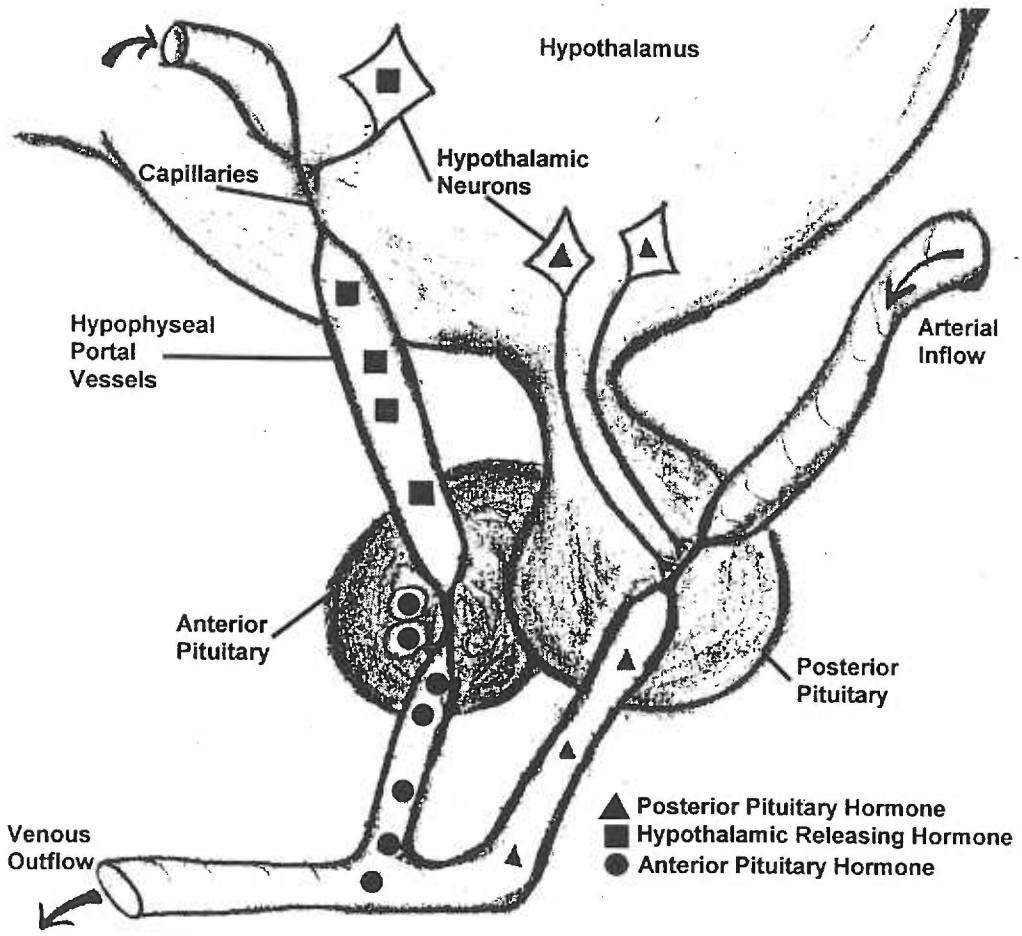


Fig. 1. The mammalian pituitary

In contrast to the posterior pituitary, the anterior pituitary is not directly connected to hypothalamic neuronal tissue. Instead, neurons in the median eminence of the hypothalamus secrete so-called releasing hormones which are transported to the anterior pituitary through the hypophyseal portal vessel system. The hypophyseal portal vessels recombine into capillaries within the anterior pituitary. Upon reaching the anterior pituitary, the hypothalamic hormones are able to modulate the expression of anterior pituitary hormone genes. This unique anatomical arrangement allows for tight regulation of anterior pituitary hormone genes by relatively low levels of hypothalamic hormones.

Hormones produced in the anterior pituitary are synthesized and secreted by one of five distinct cell types which arise in a specific temporal and spatial pattern during embryonic development. The hormones secreted by the anterior pituitary are the following: adrenocorticotropic hormone (ACTH), thyroid stimulating hormone (TSH), follicle stimulating hormone (FSH), lutenizing hormone (LH), growth hormone (GH), and prolactin (PRL). The anterior pituitary cells are known phenotypically by the hormone(s) they produce. They are corticotrophs (ACTH), thyrotrophs (TSH), gonadotrophs (FSH,LH), somatotrophs (GH), and lactotrophs (PRL).

Prolactin has provided an interesting model for the study of cell-specific, hormonally regulated gene expression. The major promoter/enhancer elements of the prolactin gene have been identified along with a cast of transactivating proteins. Although much is known about the prolactin gene and its regulated expression, the complexity of both cis and trans regulatory elements lend the prolactin gene to further study in the hopes of fully understanding all of the necessary components required for its expression.

A Brief Historical Perspective of Prolactin

The discovery and characterization of prolactin is the result of an enormous effort by many laboratories over a period of more than 50 years. Prolactin was originally identified early in the 20th century through its ability to induce lactation and mammary development in rabbits and crop sac growth in pigeons (1, 2). PRL was subsequently shown to be luteotropic, i.e., capable of inducing corpus luteum formation and action (3). Depending on the biological function of interest, PRL has also been known as lactogenic hormone or luteotropic hormone. The amino acid sequence of sheep PRL was elucidated 1969. The hormone was shown to be a protein of 199 amino acids (4). Human prolactin was isolated in 1971 (5, 6). Finally, the nucleotide sequence of PRL cDNAs from several species was determined, including rat (7), bovine (8), and human (9).

Normal and Pathological Conditions Associated with Prolactin

Normal physiological levels of PRL in vertebrates are necessary for the control over a wide range of body functions. These include the regulation of water and electrolyte balance, growth and development, metabolism, behavior, reproduction, and immunoregulation. In mammals, prolactin has been shown to reduce renal sodium and potassium excretion (10), increase intestinal water and salt absorption (11), and reduce amniotic fluid volume (12). In rats, normal prolactin levels have been associated with postnatal testes, prostate and body growth (13, 14). Prolactin is known to stimulate fetal lung phospholipid synthesis (15) and lipoprotein lipase activity in the rat (16). Normal elevated post-partum prolactin levels are thought to be involved in the stimulation of maternal behavior (17-19). Prolactin is responsible for the production of milk proteins (20), lactose (21), and milk lipids (22). Prolactin has also been shown to increase IgG and IgM antibody production (23-25) and induce macrophage activation (26) including

the production of superoxide anion (27, 28). Abnormal serum levels of PRL have been associated with a variety of disease states.

Hyperprolactinemia has been associated with many pathological conditions. Elevated serum PRL has been linked to the perturbation of reproductive events, such as amenorrhea, galactorrhea, and impotence (29). In a small number of cases, elevated serum PRL in women has been associated with a psychosomatic state of psuedopregnancy (30). PRL has also been associated with various forms of cancer. For instance, elevated PRL is believed to increase the aggressivity of colorectal tumors (31, 32) , induce the proliferation of human breast cancer cells (33), and activate malignant B lymphocytes (34) and lymphoma cells (35). PRL has also been implicated in the exacerbation of several autoimmune diseases, such as systemic lupus erythematosus (36, 37), acute experimental allergic encephalomyelitis (38), rheumatoid arthritis (39), adjuvant arthritis (40), and graft vs. host disease (41).

Prolactin is also secreted by numerous tissues outside of the anterior pituitary such as thymus (42), circulating lymphocytes (43, 44), spleen (45, 46), decidua (47, 48), myometrium (49, 50), mammary epithelial cells (51, 52), skin fibroblasts (53), and sweat glands (53). This peripheral prolactin secretion may contribute to the occurrence of some of the aforementioned pathologic disease states. The elucidation of the mechanisms involved in the regulated expression of the prolactin gene may be helpful in developing treatments for some of these prolactin associated abnormalities.

Regulation of Prolactin

Expression of PRL in the anterior pituitary is cell type specific and tightly regulated. Expression of PRL is essentially restricted to the lactotroph cell type. PRL expression can be activated by many hormones and trophic factors including the hypothalamic hormone TRH (thyrotropin releasing hormone) (54, 55), estradiol (56)

EGF (57), and insulin (58). The primary physiological inhibitor of PRL expression is the hypothalamic hormone, dopamine. Dopaminergic agonists such as ergocryptine are also known to inhibit PRL gene expression (59). Dopamine is thought to act through the dopamine D2 receptor linked to a Gαi/αo protein to decrease prolactin expression via repression of the cAMP/PKA pathway (60, 61). Conversely, agents such as forskolin (62), which increases intracellular cAMP levels, and membrane permeable cAMP analogs (63) or PKA catalytic subunit expression vectors (64) are known to stimulate expression of the prolactin gene.

The classical transducer of the cAMP-PKA signal pathway is the cAMP-response element binding protein (CREB) (65). CREB binds to a sequence specific DNA element, the cAMP response element (CRE), and is activated upon phosphorylation by PKA. However, since the PRL promoter sequence does not contain a high affinity CRE and expression of the PRL gene is not affected by the presence of a dominant inhibitor of CREB (66), CREB is most likely not directly involved in regulation of the prolactin gene by cAMP. Therefore, the cAMP- PKA signaling pathway must be transduced to the PRL gene through another route. The identification of the pathways involved in conveying the cAMP signal to the prolactin gene is critical for determining the mechanism of cAMP induced prolactin gene regulation.

The Prolactin Promoter

Expression of the prolactin gene is under control of cis DNA elements located within approximately two kilobases upstream (5') of the transcription start site. The 5' flanking sequence of the prolactin gene is divided into a proximal promoter region and a distal enhancer/promoter element. The proximal promoter sequence is located between –422 and +33 of the transcription start site. The distal enhancer sequence is located between -1831 and –1530 (Fig.2) (67-72).

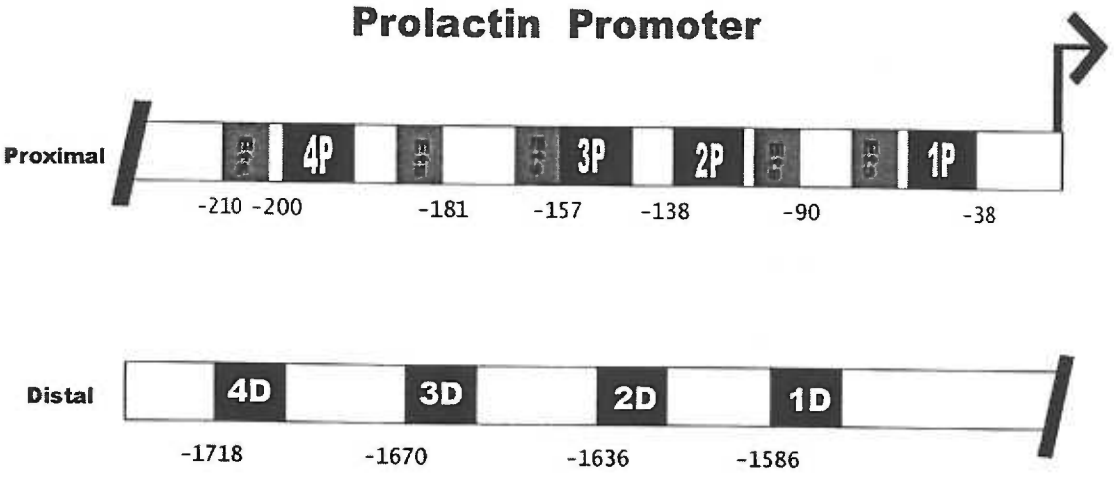


Fig.2. Schematic representation of the proximal and distal prolactin promoter/enhancer. Ets indicates putative Ets-factor binding sites. 1P-4P indicate Pit-1 binding sites in the proximal promoter. 1D-4D indicate Pit-1 binding site in the distal enhancer. Numbers represent approximate distance from transcription start site.

The proximal promoter was found to be sufficient for tissue-specific expression of reporter genes transfected into rat pituitary cell lines (73-75). However, the distal enhancer/promoter and its flanking sequence appear to be necessary to restrict the expression of PRL to the pituitary lactotrophs in transgenic mouse models (76). While each of these promoter regions is independently capable of directing low levels of

reporter gene fusion expression, both cis elements are required for a high level of synergistic expression of reporter genes in transgenic mice (76). DNAseI footprinting with pituitary extracts and mutational analysis studies identified four related non-adjacent cis-elements within both the distal and proximal PRL promoter region (73). These elements were termed 1D through 4D in the distal promoter/enhancer and 1P through 4P in the proximal promoter region.

Transactivating Factors Involved in Prolactin Expression

The discovery of related cis elements within the proximal prolactin promoter led to the identification of several transactivating factors that have been implicated in the regulation of expression of the prolactin gene. Three factors, the transcription factors Pit-1 and Ets-1 and the co-activator CBP/p300 seem to be of critical importance to the regulation of the prolactin gene. The history and characteristics of Pit-1, Ets-1, and CBP/p300 will be discussed in the following sections.

PIT-1

Multiple cis-elements in both the proximal and distal PRL promoter were found to bind a common tissue specific transcription factor known as Pit-1 or GHF-1 (77, 78). Pit-1 is a 33kd protein which contains a 160 amino acid long region that defines it as a member of the large family of POU-domain transcription activators (79). The POU domain was originally identified as a region of sequence homology between the transcription activators Pit-1, Oct-1, Oct-2, and UNC-86(**P**it, **O**ct, **U**nc). The POU domain of Pit-1 consists of two discrete, structurally independent regions: the POU-homeodomain and the POU-specific domain (80, 81). The POU-homeodomain and the POU-specific domain are separated from each other by a flexible linker region (82). The linker appears to be void of ordered structure and apparently functions only

as a physical tether between the two subdomains (83). Mutagenesis studies of Pit-1 have demonstrated that a functional cooperativity between the POU-homeodomain and the POU-specific domain is necessary for optimal DNA binding (81). Alone, the POU-homeodomain of Pit-1 is sufficient to direct binding to A/T rich DNA sequences with minimal sequence specificity (81). However, the POU-specific domain is required for high affinity, site specific binding to native Pit-1 binding sequences. In fact, the affinity of the POU-homeodomain for physiological Pit-1 responsive sequences is increased 1000-fold by the presence of the wild type POU-specific domain (81).

The crystal structure of Pit-1 binding to a DNA element has recently been resolved (83). Analysis of the structure confirmed that Pit-1 is capable of binding DNA as a homodimer. Further analysis of the crystal structure showed that each POU subdomain binds to perpendicular faces of the DNA sequence rather than opposing faces like another POU-domain family member whose structure has been solved, Oct-1 (84). This presumably allows for unobstructed access to each POU subdomain by other nuclear factors and/or co-activators.

Pit-1 expression, as determined by in situ hybridization and immunohistochemistry, has been observed in several anterior pituitary cell types. Pit-1 expression has been observed in lactotrophs, somatotrophs, and thyrotrophs (85), yet each of these cell types expresses a distinct peptide hormone. This suggests that Pit-1 is required for the expression of several anterior pituitary hormones. In addition to the prolactin gene, Pit-1 has been shown to be necessary for regulation of growth hormone (75) and thyrotropin beta subunit (TSHb) gene expression (86, 87).

Evidence of the necessity of Pit-1 expression for proper pituitary growth and function can be seen in naturally occurring mutations in both human and mouse. Mutations in the dwarf locus (dw) in the Jackson dwarf mouse result in the abnormal

development of the pituitary marked by specific hypoplasia of lactotroph, somatotroph, and thyrotroph cell types and subsequent deficiencies in their respective hormones (88, 89). A large deletion within the Pit-1 gene was ultimately found to be responsible for the Jackson dwarf phenotype (90). In humans, combined pituitary hormone deficiency syndrome has been attributed to various point mutations in the Pit-1 gene (90-92).

At least two isoforms of Pit-1 have been identified which result from differential splicing (93, 94). Pit-1b, which contains a 26 amino acid insert in the N-terminal transactivating domain, is more effective than Pit-1 at activating transcription from the growth hormone promoter, but is unable to activate transcription of a prolactin promoter-CAT reporter construct (93, 95). Pit-1b was also shown to block both basal and Ras induced activation of a prolactin-luciferase reporter in rat GH4 pituitary cells, but the same reporter construct was activated by Pit-1 in a HeLa cell transient transfection assay (96). The expression of another splice variant, Pit-1T, is restricted to the thyrotroph lineage. Pit-1T contains a 14 amino acid insertion in the transactivation domain (94). Pit-1T is capable of synergistically activating the TSHb promoter when co-transfected along with Pit-1 into Pit-1 deficient thyrotroph cell line (alpha TSH) (97). Pit-1T is also able to specifically activate the TSHb promoter but not the GH nor PRL promoters when expressed in GH3 cells (97). The Pit-1b and Pit-1T isoforms provide possible evidence for a model of tissue specific gene expression whereby the recruitment of a specific combination of several factors is necessary for the proper expression of tissue specific genes.

Since Pit-1 expression is not limited to the lactotroph cell phenotype, and is necessary for the expression of other anterior pituitary hormones, Pit-1, by itself, must not be sufficient to confer the lactotroph phenotype. Additional factors must be required

for regulation of anterior pituitary genes in the Lactotroph, somatotroph, or thyrotroph lineages.

ETS-1

Another transactivating factor important for PRL promoter activation is ETS-1, a member of the ETS super family of transcription factors. The ETS (E26 transformation specific) family was initially discovered as a Gag-Myb-Ets fusion protein in the avian erythroblastosis virus E26(v-ets). The Ets sequence was found to be responsible for eliciting erythroblastosis. Subsequently, several cellular ETS genes were identified including Ets-1 (98), Ets-2 (98), Elk-1 (99), and SAP-1 (100) to name a few. The ETS superfamily members are characterized by a related sequence of approximately 85 amino acids (the ets domain) which is responsible for sequence specific DNA binding (100, 101). The Ets domain contains three highly conserved tryptophan residues separated by 17-21 amino acids and a region of basic amino acid residues. Both the tryptophan and basic amino acid regions of Ets factors are required for binding to DNA. Although the three tryptophan residue motif is reminiscent of that found in Myb proteins, (102, 103), the structure of Ets protein DNA binding domains appears to be a unique winged helix-turn-helix motif (104). The Ets proteins bind as monomers to a 10 bp purine rich DNA domain which contains a core motif with the consensus sequence of GGAA/T (105, 106). The sequences flanking the core are variable and are required to direct the specificity of Ets protein binding. Ets-1 is known to facilitate transcription in cooperation with other transcription factors such as AP-1 in the polyoma virus enhancer (107, 108) and SP1 in the HTLV1 long terminal repeat (109).

The proximal prolactin promoter contains at least five consensus Ets binding sites [(A/G)GGAA] (Fig.2), raising the possibility that an Ets factor may play a role in the regulation of prolactin expression (110). Recent experiments have demonstrated the

involvement of Ets-1 in activating expression of the prolactin gene. Perturbation of specific Ets consensus sites within the proximal 255 base pairs of the proximal prolactin promoter is capable of reducing the prolactin gene responsiveness to TRH in rat pituitary GH3 cells (111). This indicates that an Ets factor is required for transduction of the TRH signal to the prolactin promoter. Also, transient transfection of Ets-1, but not the highly homologous Ets-2 which recognizes the same core DNA sequence as Ets-1(GGAA) (112), has been shown to enhance the expression of a prolactin promoter reporter gene construct (113). Furthermore, expression of the DNA binding domain of Ets-2 is capable of blocking both Ras and Raf induced prolactin gene expression (113). Thus, the specific Ets family member, Ets-1, appears to be directly involved in the regulated expression of the prolactin gene.

CBP/p300

The co-activator CBP/p300 is another factor which is probably important for the regulation of the prolactin gene. p300 and CBP are large, approximately 2400 amino acid, nuclear phosphoproteins which function as transcription co-activators. Co-activators are thought to operate by physically bridging the gap between DNA bound transcription factors and components of the basal transcription machinery. p300 and CBP are considered functional homologues and are usually referred to as CBP/p300. Consistent with the function of a co-activator molecule, CBP/p300 does not appear to bind directly to DNA. Instead, CBP/p300 binds to the basal transcription factor TFIIB as well as several specific transcription factors including Pit-1 and Ets-1.

p300 was first identified as a cellular target for the adenovirus transforming protein E1A (114, 115). E1A presumably induces cellular transformation by sequestering cellular proteins involved in host cell gene transcription (116). The

predominant cellular protein that associates with the N-terminus of E1A was found to be p300 (117).

CBP was initially discovered in experiments which examined the process by which extracellular signals are transduced from the second messenger cAMP. Genes induced by cAMP contain a conserved DNA element with the consensus sequence TGACGTCA known as the CRE (cAMP responsive element) (118, 119). CREB (CRE binding protein) binds to the CRE of cAMP responsive genes (120, 121). CREB is directly phosphorylated by cAMP dependent protein kinase A at a specific residue, serine 133 (122). The phosphorylation of CREB at serine 133 is able to activate transcription of CRE regulated genes. In order to identify phospho-CREB binding partners, a human thyroid lamdaGT11 library was screened with CREB which was radioactively labeled at its PKA phosphorylation site and a protein of Mr 265K was identified as CBP (123).

CBP/p300 contains distinct structural and functional features that are indicative of a transcriptional co-activator (Fig.3). p300 and CBP share 75% similarity and 63% identity over their entire sequence (124). CBP/p300 contains a centrally located bromodomain-like region (124). The evolutionarily conserved bromodomain (125, 126) of a yeast coactivator, Gcn5, has recently been shown to be directly involved in the coordination of nucleosome remodeling (127). CBP/p300 also has intrinsic histone acetyltransferase (HAT) activity (128, 129). The recent identification of HAT activity in a known transcription regulator helped to give credibility to the long held hypothesis that acetylation of histone N-terminal tails leads to transcription activation (130). The acetylation of histone tails presumably serves to modify their interaction with DNA by neutralizing the charges of specific lysine residues (131). Therefore, not only does CBP/p300 have the ability to recruit general transcription factors to a promoter, but

CBP/p300 may also directly influence chromatin structure. Finally, CBP/p300 contains three cysteine and histidine rich regions with a characteristic spacing between the residues which are thought to coordinate zinc ions in the same manner as zinc finger motifs (124). The zinc finger domains of CBP/p300 are believed to be important for forming protein-protein interactions between CBP/p300 and various transcription factors including Pit-1 and Ets-1.

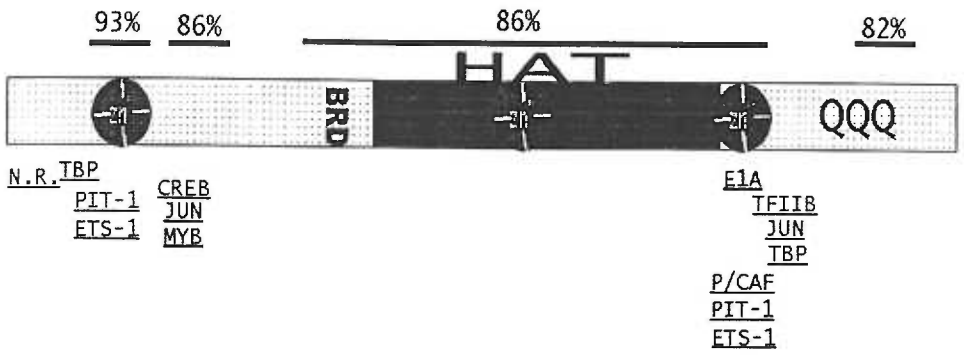


Fig.3. Diagrammatic representation of structural components of CBP/p300.
Percentages above the diagram represent the percent identity at the amino acid level of p300 and CBP. Zn = putative zinc finger domains. BRD = Bromodomain. HAT = Histone Acetyl Transferase domain. QQQ = glutamine rich domain. Items below diagram represent examples of known binding partners and their approximate binding locations.

Transactivating Factor Functional Interactions

A model for cell specific inducible gene expression depends on the precise recruitment and coordination of a specific combination of transcription factors and co-activators to the promoter sequences of active genes. Once assembled at the promoter, the transcription factor/co-activator complex is then able to recruit the basal transcription machinery to activate efficient transcription. Knowledge of the specificity of

functional and physical interactions between transactivating factors is critical for the elucidation of the mechanism of inducible gene expression. In the prolactin promoter model, several transactivating factors have been shown to specifically interact. Identification of the complete cast of transactivating factors and their specific interactions in response to extracellular signals is paramount to understanding the mechanisms involved in prolactin gene expression. The combinatorial interactions between Pit-1, Ets-1, and CBP/p300 may be important for the modulation of prolactin gene activity. The evidence for interactions between pairs of Pit-1, Ets-1, or p300 will be presented in the following discussion.

Pit-1 and Ets-1 Interactions

Pit-1 and Ets-1 have been shown to functionally cooperate to stimulate prolactin gene expression and enhance its response to various signaling pathways. Co-expression of both Ets-1 and Pit-1 in rat pituitary GH4 cells increases the Ras/Raf responsiveness of a rat reporter gene construct consisting of 425 base pairs of the proximal prolactin promoter (–425PRL) in a synergistic manner (113). Furthermore, co-expression of dominant negative forms of either Ets-1 or Pit-1(Ets-1DNA binding domain or Pit-1B, respectively) is capable of blocking the Ras/Raf induced Pit-1/Ets-1 synergy, thus implicating the necessity of a functional interaction between Pit-1 and Ets-1 for optimal Ras/Raf responsiveness of the rat prolactin gene (113). Examination of the rat proximal prolactin promoter determined the presence of a composite Pit-1/Ets-1 binding cis-element at the 3P Pit-1 binding site. A reporter construct containing multimers of this 3P site was responsive to Ras, Raf or phorbol ester treatment (132). Mutation of either the Ets site or the Pit-1 site within the 3P multimer reporter construct resulted in a decrease in responsiveness of the reporter gene to either Ras, Raf, EGF, or phorbol ester treatment (132). Thus, there are several lines of evidence which

demonstrate a functional interaction between Pit-1 and Ets-1 for hormonal activation of the prolactin gene.

Pit-1 and Ets-1 also interact cooperatively and selectively to increase the basal expression of the prolactin gene. Co-expression of Pit-1 and Ets-1 synergistically enhances the basal activity of a rat PRL reporter gene in a non-pituitary HeLa cell line (133). However, Pit-1b fails to synergistically activate basal rat prolactin promoter reporter expression when co-transfected with Ets-1 in the same system. Likewise, Pit-1 fails to synergize with Ets-2 (133). These observations demonstrate the selectivity of Pit-1 and Ets-1 activation of the rat PRL promoter.

A DNA independent physical interaction between Pit-1 and Ets-1 was also demonstrated by Bradford et al (133). They showed that GST-Pit-1 or GST-Pit-1B fusion proteins were both able to bind to ^{35}S-met-labeled Ets-1 but not to similarly labeled Ets-2. Truncation mutants of Ets-1 were used to map the regions of Ets-1 which are necessary for activity and synergy with Pit-1. (133). The region encompassing amino acids 218-312 of Ets-1 was found to be crucial for both Pit-1 binding and basal Pit-1 synergy. Interestingly, this region corresponds to an area of divergence between Ets-1 and Ets-2 and may account for the inability of Ets-2 to bind Pit-1 and activate the PRL gene.

The functional interaction between Pit-1 and a specific Ets family member, Ets-1, appears to be important for the regulation of expression of the prolactin gene in both basal and hormonally activated conditions. One mechanism which might mediate the functional interaction between Pit-1 and Ets-1 could involve the recruitment of a co-activator protein to the prolactin promoter. The following sections will discuss the known interactions between Pit-1 or Ets-1 and CBP/p300

Pit-1 and CBP/p300 Interactions

Pit-1 and CBP associate both in vivo and in vitro. CBP has been shown to co-immunoprecipitate with Pit-1 in 293 cells that were first transfected with a Pit-1 expression plasmid (134). The domains of CBP required for in vitro Pit-1 binding have been identified by GST-CBP fragment pull down assays (134). Pit-1 was found to have high affinity for either the first or third zinc finger region of CBP. Co-transfection of both Pit-1 and CBP expression plasmids into 293 cells resulted in a synergistic activation of a reporter gene construct driven by the 1P prolactin proximal promoter element (134). Furthermore, microinjection of anti-CBP antibodies into cells transfected with a gal4-Pit-1 construct abolished forskolin induced activation of a Gal4 dependent reporter gene (134). A similar result was obtained when anti-CBP antibodies were injected into cells transfected with Pit-1 and a proximal PRL 1P element driven reporter gene (134). Micro injection of CBP deletion mutants along with anti-CBP antibodies into cells expressing Pit-1 allowed for the determination of those domains of CBP that are required for the forskolin induced Pit-1 dependent activation of the 1P reporter gene construct (134). Both the HAT and third zinc finger domain of CBP were found to be necessary for the Pit-1 dependent activity (134). Pit-1 and p300 also appear to cooperate in vivo to activate the expression of a much larger PRL reporter gene construct (135). The group of Tolon et al demonstrated that the activation of a –3000PRL-CAT reporter construct was significantly enhanced by the co-expression of CBP and Pit-1 in HeLa cells (135).

The functional interaction between Pit-1 and the co-activator CBP/p300 appears to be important to the regulated expression of the prolactin gene. Moreover, the cAMP signaling pathway appears to play a direct role in the Pit-1 dependent activation of the prolactin gene by CBP. Therefore, the list of specific transactivator interactions that are

involved in the expression of the prolactin gene has been expanded to include Pit-1/Ets-1 and Pit-1/CBP/p300 interactions. This suggests the possibility of the requirement of an Ets-1/p300 interaction in the regulation of expression of the prolactin gene. The functional interaction between Ets-1 and CBP/p300 has also been investigated and will be discussed in the following sections.

Ets-1 and CBP/p300 Interactions

Although the functional interaction between Ets-1 and CBP/p300 has not yet been seen for the prolactin gene, the interaction has been observed in the context of two physiological relevant genes; the CD13/APN and the stromelysin genes. Initial evidence for an interaction between Ets-1 and CBP was observed by the group of Yang et al who were studying regulation of the hematopoietic amino peptidase N (CD13/APN) gene (136). Transcriptional activation of the CD13/APN gene in hematopoietic cells is dependent upon the functional interaction of Ets-1 and Myb (137). Since CBP has previously been shown to function as a co-activator for Myb (138), Yang et al sought to investigate the possibility of an Ets-1 and CBP interaction in the modulation of the CD13/APN gene. Yang et al initially found that expression of a CD13/APN luciferase reporter in KG1a myeloblastic cells was inhibited in the presence of the adenoviral E1A protein. This finding raised the possibility of a direct involvement of CBP/p300 in the Ets-1 dependent expression of the CD13/APN gene. An Ets-1 and CBP/p300 link was further established by observing that E1A could also inhibit a version of the CD13/APN reporter gene lacking a functional Myb dependent DNA element. This indicated that E1A was possibly targeting the Ets-1 component of the reporter gene and supported the hypothesis that CBP/p300 functions as a co-activator of Ets-1. Yang et al also observed E1A inhibition of Gal4-Ets-1 induced activation of a gal4 dependent reporter gene. Furthermore, Ets-1 was found to potentiate both CBP and p300 activation of the

CD13/APN reporter. Finally, Yang et al demonstrated in vitro binding of CBP/p300 to Ets-1. They used GST-pull down and co-immunoprecipitation experiments to show that Ets-1 is capable of binding to either the first or third zinc finger domains of CBP/p300. The experiments performed by Yang et al provided strong evidence of a functional interaction between Ets-1 and CBP/p300 in the modulation of the hematopoietic CD13/APN gene.

The Human stromelysin promoter is also activated in a synergistic manner by Ets-1 and p300 (139). The stromelysin promoter is dependent on a palindromic Ets site and an AP1 site (140, 141). The Ets site in the stromelysin promoter can be bound and activated by either Ets-1 or Ets-2 (142). Therefore, Jayaraman et al sought to determine if CBP/p300 could interact with an Ets factor and co-activate Ets dependent stromelysin promoter activity. To this end, they utilized HeLa cells which were transiently transfected with a reporter construct containing the human stromelysin promoter. They found that co-transfection of both Ets-1 and p300 resulted in a 25 fold synergistic activation of the stromelysin promoter construct. Jayarman et al also used GST-pull down experiments to determine that Ets-1 could bind to either the N or C terminal regions of CBP/p300. These regions of CBP/p300 correspond to the same first and third zinc finger domains which were shown to bind Ets-1 by Yang et al. Together, the experiments of Yang and Jayaraman provide substantive evidence that Ets-1 makes a direct functional interaction with the co-activator CBP/p300 and this interaction may be involved in the regulation of at least two physiologically relevant genes; the CD13/APN and the Human stromelysin genes.

The experiments described above describe the interactions of combinations of Pit-1, Ets-1, or p300 in the involvement of specific gene expression. Pit-1/Ets-1 and likewise, Pit-1/p300 have been shown to be involved in the regulation of the prolactin

gene. An Ets-1/p300 interaction has not yet been demonstrated in the context of the prolactin gene, but given their interaction in the regulation of other genes, it is very likely that they do interact and facilitate the expression of the prolactin gene. The complete cast of transactivating factors and the specific interactions which are required for regulated cell specific expression of the prolactin gene remains to be discovered. At a minimum, it is likely that a tripartite complex of Pit-1/Ets-1/p300 serves to coordinate the regulated expression of the prolactin gene with various extracellular signals.

The work presented in this thesis is divided into two chapters. Chapter 2 represents work performed exclusively by Jeffrey Lauten in the laboratory of Richard Maurer. This chapter explores the involvement of the transcriptional co-activator p300 in activation of expression of the prolactin promoter. Chapter 3 is based on a manuscript which has been submitted for publication. It represents a collaborative work by Paul Kievit and Jeffrey Lauten in the laboratory of Richard Maurer. Experiments in figures 1 and 3 were performed exclusively by Jeffrey Lauten and the Gal4-p300 expression vector used in figure 5 was constructed by Jeffrey Lauten. This chapter explores a possible role for the mitogen activated protein kinase (MAPK) in mediating cAMP activation of the prolactin promoter.

CHAPTER 2

Analysis of p300 Involvement in Prolactin Gene Expression

INTRODUCTION

The proposed model for cell specific, hormonally-regulated gene expression involves the assemblage of discrete combinations of transactivating factors at the promoter. The determination of the specific combinations of transactivating factors that are required for the modulation of expression of certain genes is important for understanding both the mechanism of activation of the particular gene and the mechanisms of transcriptional activation in general. The prolactin gene, with its complexity of both cis and trans factors, serves as a good model for the study of cell specific, hormonally-regulated gene activation. The functional interaction between several transactivating factors has been implicated in the modulation of expression of the PRL gene. The transcription factors Pit-1 and Ets-1 and the co-activator CBP/p300 are believed to be of particular importance for the regulation of PRL gene expression. Several lines of evidence have shown that the interaction between pairs of these factors is important for the regulation of PRL expression. The functional interaction between Pit-1 and Ets-1 has been shown to be critical for the expression of a PRL reporter gene construct (113) and a reporter gene consisting of multimers of the proximal PRL

promoter 3P Pit-1 binding site (132, 133). CBP/p300 has been shown to interact with Pit-1 both in vitro and in vivo and to enhance the expression of a PRL reporter gene (135), and a reporter gene containing a multimer of a Pit-1 binding site (134). While not yet observed in the context of the PRL gene, the interaction between Ets-1 and CBP/p300 has been observed in at least two other genes, namely theCD13/APN (136) and the stromelysin (139) promoters. Since the complete cast of transactivating factors involved in the regulation of the PRL gene has yet to be determined, it is likely that the combination of Pit-1, Ets-1, and p300 is directly involved in its regulation. Given the possibility that large, multimeric complexes of transcription factors and co-activators are necessary for the coordination of various intracellular signaling pathways with cell specific gene expression and the physical and functional interactions of Pit-1, Ets-1, and CBP/p300 which have been observed by others, we propose that a complex of Pit-1, Ets-1, and p300 is likely involved in the regulation of expression of the PRL gene. Therefore, we sought to determine if Pit-1, Ets-1, and p300 could act in combination to modulate the activity of the PRL gene. Additionally, in an effort to determine the mechanism by which hormone signals regulate PRL gene expression, we further investigated the intracellular signaling pathways which affect the Pit-1/Ets-1/p300 dependent expression of the PRL gene.

MATERIALS AND METHODS

Cell culture and transient transfections

F9, 293, and COS-1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum. NIH3T3 cells were cultured in DMEM supplemented with 10% calf serum. GH3 cells were cultured in DMEM supplemented with 15% horse serum and 2.5% fetal bovine serum. For transient transfections, cells were split into 6 well tissue culture dishes at an approximate density of 500,000 cells per well. Cells were allowed to grow overnight. 2 ug total plasmid DNA was added to the cells with Lipofectamine reagent according to manufacturer's protocol. All assays included 100 ng CMV-β-Galactosidase expression vector to assess transfection efficiency. Cells were incubated with DNA and Lipofectamine for 3 to 16 hours in 1 ml opti-mem II medium. 1 ml DMEM containing 2 x serum was added and cells were further incubated for 6 to 16 hours. Cells were washed once in PBS, lysed in passive lysis buffer (Promega, Madison WI), and analyzed for luciferase activity.

DNA dependent Avidin-Biotin Complex assay.

A 255 bp biotinylated prolactin DNA element was generated via PCR. PCR primers consisted of the following sequences; biotin-CAA CAG TAC CGG AAT GAA GC and CAA TTC GAG CTC GGT ACC CG. The biotinylated double stranded PRL DNA was purified on a 1% agarose gel and isolated by QiaexII gel extraction (Qiagen, Santa Clara CA). The biotinylated DNA was bound to Streptavidin conjugated Dynabeads (Dynal Oslo, Norway) and washed in B&W buffer containing 10mM Tris-HCl (Ph7.5), 1 mM EDTA, and 2 mM NaCl according to manufacturer's protocol. Recombinant proteins, which were graciously provided by Shall Jue and Ronald Lickteig from the

Maurer lab, were added to the streptavidin bound PRL DNA in binding buffer consisting of 150mM NaCl and 5mM MgCl2 and incubated at 4°C for 30 minutes in the presence of 5ug sheared salmon sperm DNA. The protein complexes bound to the beads were magnetically separated and washed 5 x in binding buffer. Samples were then subjected to poly acrylamide gel electrophoreses and transferred onto Immobilon membranes (Millipore Corp, Bedford, MA) at 0.2 mA overnight. Membranes were then incubated sequentially with the appropriate antibodies; rabbit-anti-Ets-1/Ets-2, 1:5000 dilution, rabbit-anti-p300(N15) 1:1000 (Santa Cruz biotechnology, Santa Cruz, CA), or rabbit-anti-Pit-1 serum 1:10,000 dilution for 1 hour at room temperature. Membranes were washed 3 x 10 minutes in TBS-Tween 20 + 5% nonfat dry milk (blocking buffer). Membranes were incubated with HRP (horse radish peroxidase) conjugated 2° antibody, goat-anti-rabbit IgG 1:5000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA), washed 3 x 10 minutes in blocking buffer and visualized using enhanced chemiluminescence (NEN, Boston, MA).

p300 deletion mutants

p300 deletion mutants were created via PCR and subcloned into PCDNA3. In each case, the PCR primers used for the termini of p300 were the following; N terminus p300 AGA CCC AAG CTT ACC ATG GCC, C terminus GAT GCA TGC TCG AGC GGC CGC. Internal primers containing an engineered KPN1 site were used to generate each deletion. The engineered KPN1 site resulted in the following amino acid substitutions at the resulting conjoined wild type sequences: ΔHAT KL/GT, ΔZN1 LV/GT, ΔZN3 HL/GT. Each PCR product was subcloned into PCDNA3 individually and sequenced to determine accuracy. Each fragment was then ligated back into PCDNA3 as a Hind3/KPN1 and KPN1/XHO1 fragment. PCR primers for each deletion are as follows:

ΔHAT; TAC CCG TGA ggt acc CCG GTT ATA TAA CCA GGC and GAC AAG CAC ggt acc TTC TCT TCA CTC CG, *ΔZN1*; CTT GTC AGC ggt acc AAG GAG AAC AAG C and CAT GAT TGT ggt acc TGT CTC CCC CTC, *ΔZN3*; AGA GAA CTC ggt acc CTT GTC CCT TGC and GTG CCG TTC ggt acc AAC ATC AAG CAG AAG C. ΔZN1/ZN3 was generated by ligating a BSTEII/KPN1 fragment from ΔZN3 into the ΔZN1 construct. Expression of each deletion mutant was verified by western blotting cell lysates as described above and probed with an anti-FLAG epitope antibody (SIGMA, St. Louis MO) and a HRP conjugated anti mouse IgG secondary antibody (Santa Cruz Biotechnology, Santa Cruz CA)

RESULTS

Pit-1 and Ets-1 synergistically activate gene expression

Previous reports have provided evidence that Pit-1 and Ets-1 can cooperate to activate expression of the PRL promoter (113, 132, 133, 143). We have used transient transfection assays to confirm the functional interaction of Pit-1 and Ets-1. Mouse F9 cells were transiently transfected with the 4x3P-luciferase reporter construct and mammalian expression plasmids encoding Pit-1 and/or Ets-1 (Fig.1). The 4x3P-luciferase reporter contains 4 sequential repeats of the composite Pit/Ets 3P site from the rat proximal prolactin promoter and disruption of either the Pit-1 or Ets site has been shown to diminish the reporter gene's responsiveness to various activators (132) . We chose to test this reporter gene because it is a simplified version of the prolactin promoter which contains only Pit-1 and Ets binding sites. Therefore, any increase in

reporter gene activity with the transfection of Pit-1 and Ets-1 should be due solely to the presence of the added Pit-1 and/or Ets-1. When neither Pit-1 nor Ets-1 was expressed, there was a low level of reporter gene activity. When Pit-1 was expressed alone, a 24-fold activation of the reporter gene was observed. When Ets-1 was expressed alone, a 4-fold activation resulted. When both Pit-1 and Ets-1 were co-transfected together, the resultant reporter gene activation increased to 90-fold over background levels. Therefore, Pit-1 and Ets-1 synergistically activate the 4x3P-luc reporter gene in F9 cells confirming the results of previous studies.

We next tested if Pit-1 and Ets-1 were capable of synergistically activating expression of a more physiologically relevant example of the prolactin promoter. We transiently transfected a –255PRL-luciferase reporter gene construct along with Pit-1 and/or Ets-1 expression plasmids into a variety of heterologous cell types (Fig.2). The –255PRL-luc reporter contains all of the proximal PRL Pit-1 binding sites as well as five putative Ets factor binding sites. When transfected into NIH/3T3, 293, or COS-1 cells, the –255PRL-Luc reporter exhibited a low level of background activity. When either Pit-1 or Ets-1 was introduced, a modest increase in luciferase activity was observed. However, when Pit-1 and Ets-1 plasmids were co-transfected, luciferase activity increased in a synergistic manner. The luciferase reporter activity increased 25-fold, 59-fold, and 23-fold over background when NIH/3T3, 293, and COS-1 cells, respectively, were co-transfected with Pit-1 and Ets-1 expression vectors. Therefore, Pit-1 and Ets-1 can synergistically activate the –255PRL-luc reporter gene construct in a variety of non-pituitary cell types.

p300 enhances Pit-1/Ets-1 dependent gene activation

Since previous reports have shown p300 to cooperatively activate gene expression with Pit-1 (134, 135) or Ets-1 (136, 144), we reasoned that p300 would further enhance Pit-1/Ets-1 dependent reporter gene activation. We used transient transfection of F9 cells with the 4x3P-luc reporter gene construct to demonstrate that p300 is capable of enhancing Pit-1/Ets-1 dependent gene activation (Fig.3). The 4x3P reporter was chosen because it contains only Pit-1 and Ets binding sites and any activity seen with the addition of p300 can likely be attributed to its interaction with Ets-1 and Pit-1. The composite Pit-1/Ets 4x3P-luciferase reporter gene was activated 24-fold by Pit-1, 4-fold by Ets-1 and 2.9-fold by p300. When all three plasmids were co-expressed, the luciferase activity was increased to approximately 137-fold over background. Therefore, this data shows that p300 is capable of enhancing Pit-1/Ets-1 dependent expression of the 4x3P-Luc reporter gene in f9 cells.

Having observed that p300 is capable of enhancing the Pit-1/Ets-1 dependent expression of the relatively simple 4x3P-luciferase reporter gene, we next assessed the ability of p300 to augment the expression of a more complex reporter gene, the -255PRL-luciferase reporter construct (Fig.4). We transiently transfected the –255PRL reporter along with combinations of Pit-1, Ets-1, and p300 expression plasmids into several heterologous cell lines and measured luciferase activity. When NIH/3T3 cells were transfected with p300 and Pit-1, Ets-1 or Pit-1 + Ets-1, only a modest increase in reporter gene activity was observed (Fig.4a). Expression of p300 in 293 cells resulted in a similar, modest, approximately 1.3-fold enhancement of luciferase activity over that of the combination of Pit-1 and Ets-1(Fig. 4b). Transfection of p300 into COS-1 cells resulted in a somewhat greater enhancement of luciferase activity than was observed in the other two cell types (Fig4c). These results provide evidence that p300 is capable of

modestly enhancing Pit-1/Ets-1 dependent gene expression of a –255PRL reporter construct in a several non-pituitary cell lines.

p300 Domain requirements

CBP/p300 is a very large molecule containing several distinct structural and functional domains. It is possible that only one or several specific domains of CBP/p300 is/are required for the Pit-1/Ets-1 dependent enhancement of expression of the prolactin gene. In order to determine if certain domains of p300 are necessary for its Pit-1/Ets-1 dependent activity, we constructed several deletion mutants of p300 and assessed their activity in transient transfection assays (Fig.5). Since Pit-1 and Ets-1 have both been shown to bind to the first and third zinc finger domains of p300 (134, 136) we chose to construct p300 mutants which lacked these zinc finger domains (Δzn1, Δzn3, Δzn1/3). Given the possibility that p300's primary function as a co-activator may involve its histone acetyltransferase (HAT) activity, and removal of this domain may prevent p300 from functioning as a co-activator, we also constructed a deletion mutant lacking the entire HAT domain (ΔHAT). Each of the p300 deletion mutants was then tested in transient transfection experiments with the –255PRL reporter to assess its activation capabilities. Transfection with the ΔHAT mutant into 293 cells did not activate the PRL promoter more than Pit-1 and Ets-1 alone. This is possibly due to the poor expression of the mutant protein (Fig.5c). There was no obvious difference between the activity of the Δzn1, Δzn3 or Δzn1/3 mutants and that of wild type p300. The p300 deletion mutants showed a similar pattern of PRL gene activation in COS-1 cells (Fig5d). Δzn1 and Δzn1/3 appeared to have no obvious difference in activity when compared to wild type p300. The Δzn3 mutant showed increased activity which did not appear to be a function of increased expression levels of the mutant. ΔHAT had no more activity than Pit-1 and

Ets-1 alone but was also expressed poorly in this cell type (Fig6e). Ideally, in this type of experiment, one or more of the domain deletion mutants will exhibit a loss of function phenotype. For example, a recent report by Kraus et al (145) demonstrated that individual domains of p300 were required for the activation of transcription of chromatin templates. Unfortunately, since the activation seen by wild type p300 in our transient co-transfection assays was typically less than 2-fold above Pit-1/Ets-1 activation, a loss of function phenotype was difficult to assess. Therefore, these experiments were not able to definitively demonstrate that any one particular domain of p300 is responsible for the modest Pit-1/Ets-1 dependent enhancement of expression of the PRL gene.

The adenovirus E1A protein blocks p300 associated activation of the prolactin gene

Since the contribution of p300 to Pit-1 and Ets-1 synergy seen thus far has been small and inconsistent, we wanted to determine if endogenous p300 may play a role in the activation of the prolactin gene. To this end, we took advantage of the p300-specific binding capabilities of the adenovirus 12S E1A protein. E1A has been shown to specifically bind to p300 and inhibit its activity (146, 147). Therefore, we used E1A in transient transfection assays in an effort to block endogenous p300 effects on Pit-1 and Ets-1 synergy. The effect of E1A on Pit-1 and Ets-1 activity was first examined in F9 and COS-1 cells (Fig.6) using a Gal4 dependent reporter gene system. The 5xGal4-luc reporter gene contains 5 sequential repeats of the Gal4 binding element linked to a luciferase reporter gene. We have previously shown that p300 is capable of enhancing Gal4-Pit-1 or Gal4-Ets-1 fusion plasmid dependent activation of this reporter system (data not shown). Therefore, we chose this system to assess whether E1A is capable of blocking Pit-1/p300 or Ets-1/p300 activity. F9 cells were transfected with the 5xGal4-luciferase reporter and Gal4-Ets-1, p300, and E1A expression vectors (Fig.6a). Based

on the experiments performed by Lunblad et al (146), we chose to initially test 1.0 ug of E1A expression vector in these transfection assays. Gal4-Ets-1 had little activity when expressed alone. Luciferase activity was substantially activated by the co-expression of p300 and Gal4-Ets-1. The introduction of E1A resulted in a 6-fold reduction of the p300 dependent Gal4-Ets-1 luciferase activity indicating that the Ets-1/p300 activity in F9 cells is sensitive to inhibition by E1A. COS-1 cells were also transiently transfected with the Gal4 dependent reporter gene construct and either Gal4-Pit-1 or Gal4-Ets-1 fusion, p300, and E1A expression plasmids (Fig6b). Gal4-Pit-1 had a low level of background luciferase activity. The addition of E1A had essentially no effect on the Gal4-Pit-1 activity. In this case, E1A may be incapable of further lowering the already low level of luciferase activity induced by the Gal4-Pit-1 expression vector or perhaps the activity of Gal4-Pit-1 may be insensitive to inhibition by E1A. The omission of a Gal4 DNA binding domain control expression vector in this experiment prohibits further speculation about the activity of E1A with regards to Gal4-Pit-1 induced gene expression. The addition of p300 and Gal4-Pit-1 caused a 4-fold increase in luciferase activity and E1A effectively blocked this activation. Thus, Gal4-Pit-1/p300 induced gene expression was effectively blocked by the expression of E1A. Gal4-Ets-1 had a higher background activity than the Gal4-Pit-1 construct in this system. The addition of E1A to Gal4-Ets-1 resulted in a reduction in Gal4-Ets-1 induced reporter gene activity . This indicates that Gal4-Ets-1 activity with the 5xGal4-luc reporter construct in COS-1 cells may be at least partially dependent on an E1A sensitive component. Expression of p300 modestly enhanced the Gal4-Ets-1 activity, but the addition of E1A effectively blocked any enhancement due to p300. The addition of E1A appeared to reduce luciferase activity to below that of Gal4-Ets-1 and E1A, but statistical analysis revealed that the two conditions, Gal4-Ets-1/E1A vs. Gal4-Ets-1/E1A/p300, were not significantly different ($p>0.05$). Therefore, E1A is

able to inhibit p300 dependent Pit-1 or Ets-1 activity of a Gal4 dependent reporter gene construct in two non-pituitary cell lines.

The previous experiment was performed under the assumption that the ability of E1A to inhibit Pit-1 or Ets-1 dependent gene expression is strictly dependent on its interaction with and sequestration of CBP/p300. However, E1A is also known to bind to several other cellular proteins, most notably the retinoblastoma gene product, pRB (148), and its related family members p107 and p130 (149). The inhibitory E1A effects seen above could, therefore, be the result of E1A's interaction with pRB and not CBP/p300. To test whether E1A inhibits expression of the prolactin promoter in a CBP/p300 dependent manner, we examined the effects of wild type E1A vs. a mutant form of E1A on the Pit-1/Ets-1 dependent expression of the proximal prolactin promoter in COS-1 cells. The mutant form of E1A (E1A-R2G) contains a single (arginine to glycine) amino acid substitution, and has been shown to possess a decreased affinity for binding to CBP/p300 (146, 150) but is still able to bind to pRB (150). The inhibitory dose responses of wild-type E1A and E1A-R2G were directly compared in COS-1 cells (Fig. 7). COS-1 cells were transfected with the –255PRL-Luc reporter, Pit-1 and Ets-1 expression vectors and increasing amounts of each type of E1A expression vector (ranging from 0.8ng to 2.5ug). The addition of Pit-1 and Ets-1 resulted in a 20-fold activation of the –255PRL reporter gene activity (data not shown). The two highest concentrations (bar 6 and 7) of wild type E1A were able to completely inhibit Pit-1/Ets-1 induced PRL promoter expression, while only the highest concentration (bar 13) of the E1A mutant was able to completely repress PRL promoter expression. 20 ng of wild-type E1A was able to inhibit Pit-1/Ets-1 dependent PRL promoter expression more than 60% while the luciferase activity in the presence of 20 ng E1A-R2G was equal to Pit-1/Ets-1 alone (bar 4 vs. 10 with asterisks). The inability of the mutant E1A to inhibit PRL

promoter expression with equal efficacy as the wild-type E1A indicates that E1A may inhibit Pit-1/Ets-1 dependent expression of the prolactin promoter in a CBP/p300 dependent manner in COS-1 cells. Another mutant form of E1A exists that has been determined to bind to CBP/p300 but not pRB (Y47/928H) (150). Using this mutant form of E1A or the double mutant (R2G+Y47/928H) in a similar assay would be helpful in definitively determining if the Pit-1/Ets-1 activity inhibited by wt E1A is due exclusively to a CBP/p300 dependent activity or if another cellular activity possibly involving pRB is disturbed by the expression of E1A. Unfortunately, these reagents were not available for testing. It should be noted that these experiments were interpreted with the assumption that there were equivalent levels of expression of the E1A wild type and E1A R2G mutant. However, this may not be the case as the expression levels of the E1A proteins were not measured. In order to confidently state that the interpretation of these results is accurate, the relative expression levels of each E1A isoform must be determined by western blot experiments using specific E1A antibodies. These E1A experiments offer evidence that the Pit-1/Ets-1 synergistic activation of the prolactin promoter is dependent upon endogenous CBP/p300 or an associated factor.

In the preceding experiments, we have begun to examine the involvement of p300 in the basal expression of the prolactin promoter, but we have not yet addressed its involvement after hormonal activation. It has long been known that expression of the prolactin gene can be activated by cAMP (59, 151) or EGF (57), but the exact mechanisms of activation remain controversial. There is evidence that cAMP acts through a PKA (152, 153) signaling cascade whereas EGF signals through a MAP kinase dependent pathway (154) to activate the prolactin gene. The precise activation steps downstream of MAPK or PKA are not known. One possible mechanism for the activation of the prolactin gene by distinct signaling pathways is that different signaling

pathways recruit different combinations of co-activator proteins to the promoter. Recently, Xu et al (134) has provided evidence for this model when they found that a reporter gene containing multimers of a Pit-1 binding site utilizes the activity of different co-activator domains in response to signaling by growth factors or cAMP. In light of this finding, we assessed the involvement of endogenous CBP/p300 in expression of the proximal prolactin promoter in response to activation by either forskolin and EGF. Again, we used the adenovirus 12S E1A protein to inhibit CBP/p300 dependent activities in response to either forskolin or EGF. The amount of E1A used in these assays was determined from dose response data involving inhibition by wild type E1A vs. E1A-R2G. As in COS-1 cells, 20 ng of wild type 12SE1A effectively inhibited –255PRL expression while E1A-R2G had little or no effect. Therefore, 20 ng of E1A was used in each of the following experiments. GH3 cells were transfected with the –255PRL-Luc reporter gene construct and a 12S E1A expression plasmid (Fig.8). 24 hours later, cells were treated with either FSK or EGF and harvested after 6 hours. FSK and EGF activated –255PRL reporter gene activity by 7.6-fold and 3.9-fold, respectively. E1A did not inhibit basal reporter gene activity ($p>0.05$). E1A decreased FSK activation by 1.7-fold. E1A decreased EGF activation 2.6-fold. EGF induced gene activation was substantially inhibited by expression of E1A (1.5-fold activation) while FSK induced reporter gene activity was only partially inhibited in the presence of E1A (4.3-fold activation).

The prolactin gene has also been shown to be responsive to both Ras (155) and PKA (156). Since Ras and PKA lie downstream of the EGF and FSK signaling pathways, we investigated whether E1A could exhibit effects similar to those seen with FSK and EGF on expression of the prolactin promoter in response to activation by PKA or Ras. GH3 cells were transfected with the –255PRL-luc reporter gene, a 12S E1A

expression plasmid, and expression plasmids encoding the catalytic domain of PKA or a constitutively active form of Ras(V12Ras) (Fig.9). The –255PRL-Luc reporter was activated both by PKA (2.8-fold) and V12Ras (7.7-fold). Again, E1A had no significant inhibitory effect (p=0.2) on basal –255PRL luciferase activity. PKA activation was only partially inhibited by E1A. V12Ras activation, on the other hand, was substantially inhibited by E1A. In the presence of E1A, V12Ras induced luciferase activity was not significantly different than basal activity (p=0.88), while PKA induced luciferase activity remained significantly different than basal activity (p=<0.05).

p300 binds a prolactin promoter DNA element in a Pit-1 and/or Ets-1 dependent manner in vitro

Since p300 appeared to enhance Pit-1 and Ets-1 dependent PRL reporter gene expression and E1A was able to inhibit prolactin gene expression by a presumably CBP/p300 dependent mechanism, we sought to determine the relative affinity of p300 for binding to the prolactin promoter in the presence of Pit-1 and/or Ets-1. To assess p300's binding characteristics, we performed a DNA-dependent avidin-biotin complex assay with a biotinylated –255PRL DNA element and recombinant p300, Pit-1, and Ets-1 proteins (Fig.10) as described above. Recombinant p300 alone did not appreciably bind to the biotinylated PRL element . However, p300 did bind to the PRL promoter in the presence of either recombinant Pit-1 or Ets-1(lane 5 and 6, top row). p300 also bound to the PRL promoter in the presence of both Pit-1 and Ets-1 (lane 7, Top row). p300 did not appear to bind preferentially to the PRL promoter with both Pit-1 and Ets-1 vs. Pit-1 alone or Ets-1 alone. This data suggests that p300 is capable of binding in vitro to a prolactin promoter DNA element in a Pit-1 and/or Ets-1 dependent manner.

Fig.1 Pit-1 and Ets-1 synergistically activate gene expression. Mouse F9 cells were transiently transfected with a 4x3P-luciferase reporter containing 4 sequential 3P composite Pit-1/Ets binding sites from the rat proximal prolactin promoter linked to a firefly luciferase gene. The amount of reporter gene expression was measured by relative luciferase light units +/- S.E.M. normalized to an internal CMV-beta-galactosidase control expression plasmid.

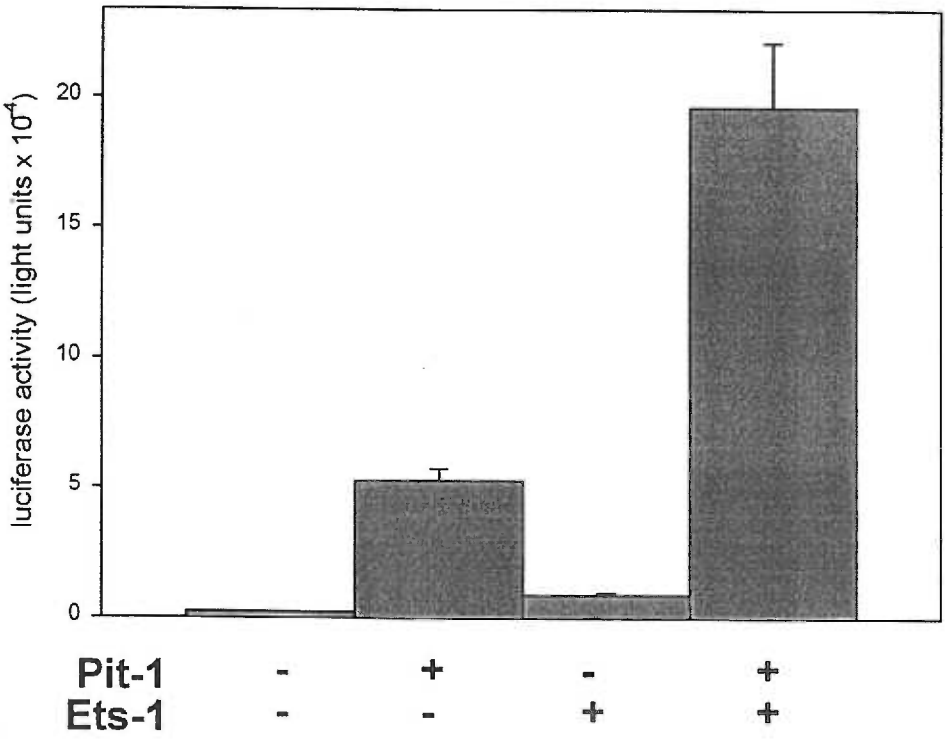

luciferase activity (light units x 10^-4)
20
15
10
5
0
Pit-1 - + - +
Ets-1 - - + +

Fig.2. Pit-1 and Ets-1 synergistically activate the –255PRL-Luc reporter construct.(A)Mouse NIH/3T3, (B)Human 293, and (C)African green monkey COS-1 cells were transiently transfected with a –255PRL-luciferase reporter gene construct and Pit-1 and/or Ets-1 mammalian expression plasmids. The amount of reporter gene expression was measured by relative luciferase light units +/- S.E.M. normalized to an internal CMV-beta-galactosidase control expression plasmid.

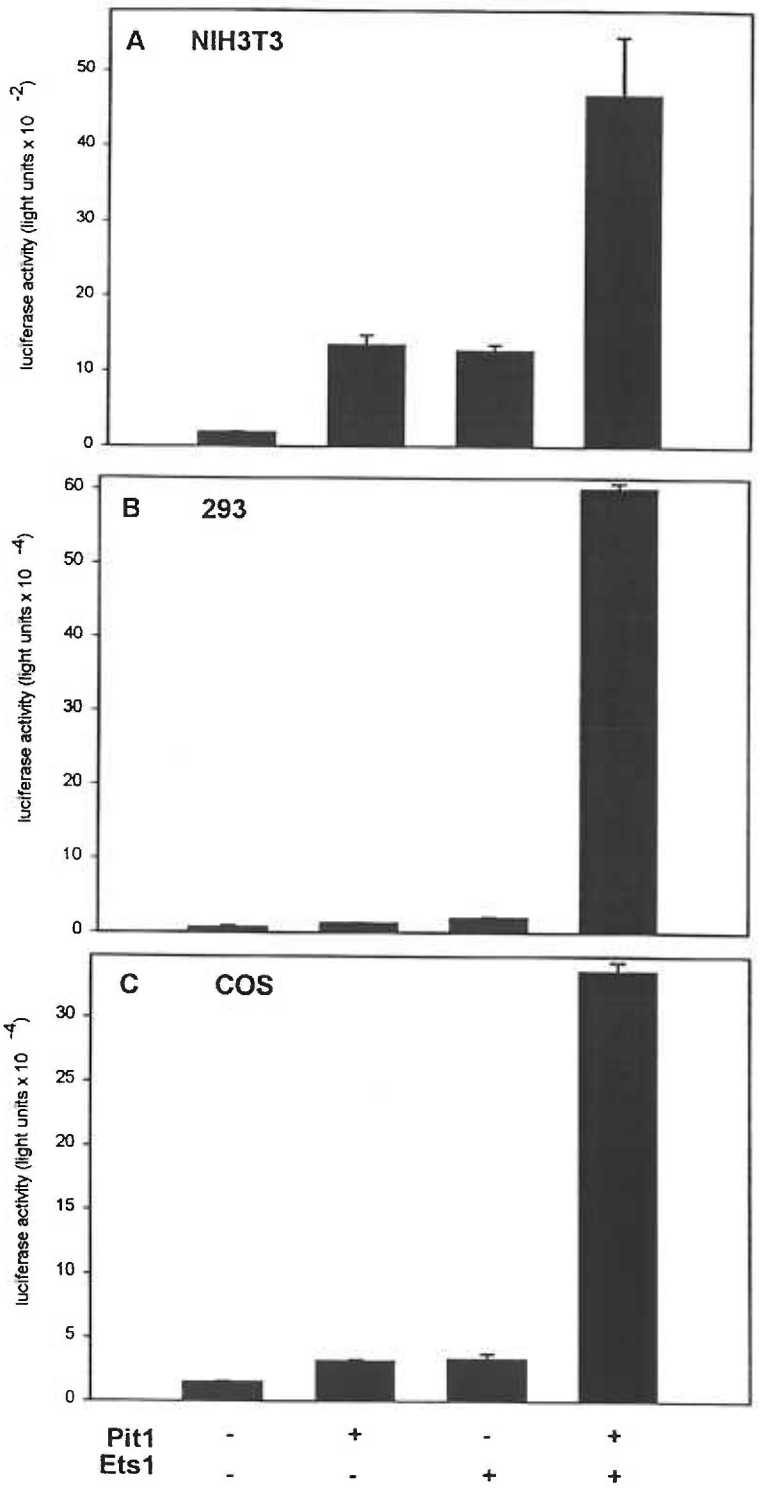

A NIH3T3
luciferase activity (light units x 10 -2)
50
40
30
20
10
0
B 293
luciferase activity (light units x 10 -4)
60
50
40
30
20
10
0
C COS
luciferase activity (light units x 10 -4)
30
25
20
15
10
5
0
Pit1 - + - +
Ets1 - - + +

Fig.3. p300 enhances Pit-/Ets-1 synergy. F9 cells were transiently transfected with a reporter gene construct containing multimers of the proximal prolactin promoter 3P site linked to a luciferase reporter gene(4x3P-Luc) and combinations of Pit-1 Ets-1 and p300 expression vectors as indicated. The amount of reporter gene expression was measured as luciferase light units normalized to an internal CMV-βgalactosidase control expression plasmid and expressed as Relative Light Units(RLU)+/- S.E.M. Numbers at top of bars indicate fold activation of luciferase expression over reporter plasmid alone.

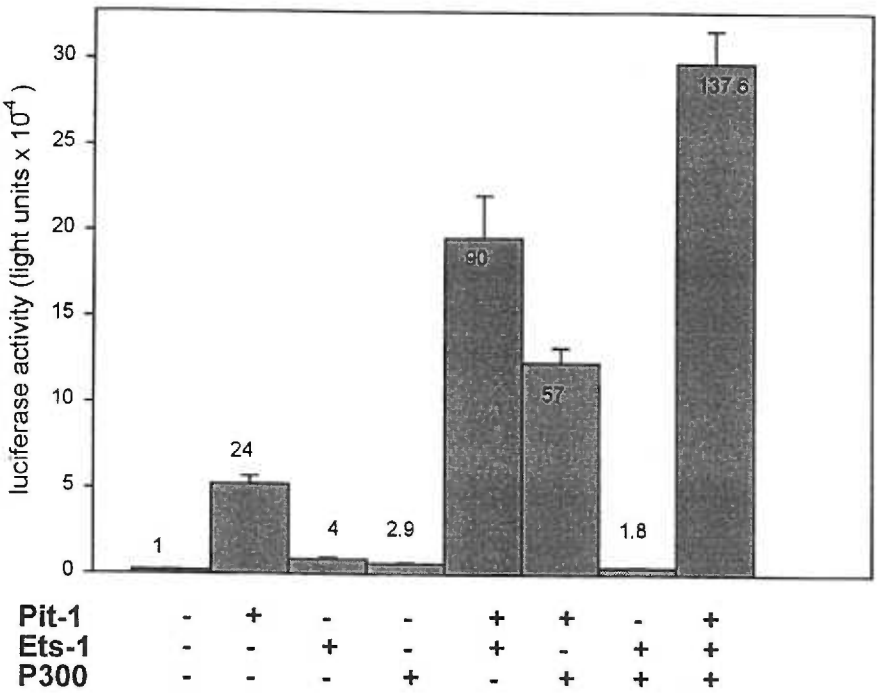

luciferase activity (light units x 10^4)
30
25
20
15
10
5
0
1
24
4
2.9
90
57
1.8
137.6
Pit-1 - + - - + + - +
Ets-1 - - + - + - + +
P300 - - - + - + + +

Fig.4. p300 enhances Pit-1/Ets-1 synergistic activation of the –255PRL-Luc reporter gene construct. (A)NIH/3T3, (B)293, and (C)COS-1 cells were transiently transfected with the –255PRL reporter and combinations of Pit-1, Ets-1, or p300 expression plasmids as indicated. The amount of reporter gene expression was measured by relative luciferase light units +/- S.E.M. normalized to an internal CMV-beta-galactosidase control expression plasmid.

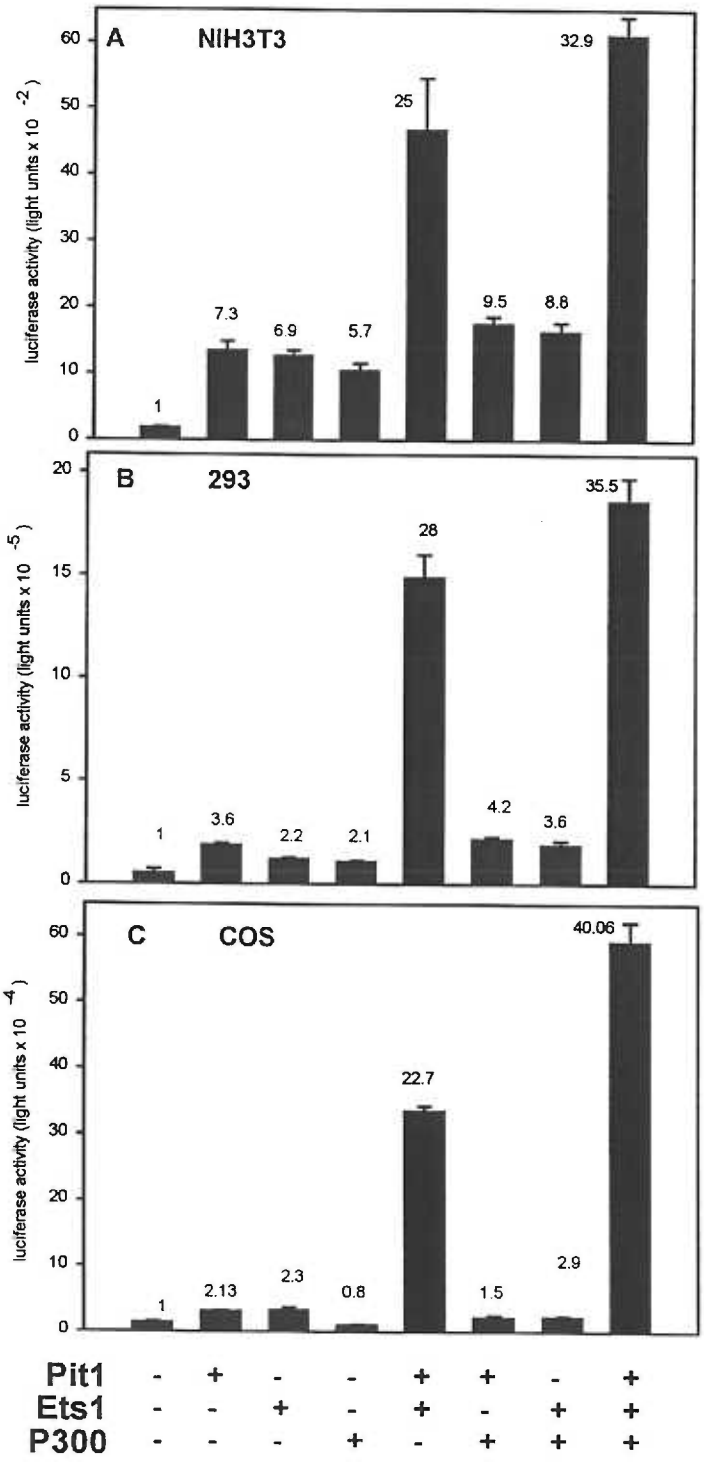

A
NIH3T3
luciferase activity (light units x 10 -2)
1
7.3
6.9
5.7
25
9.5
8.8
32.9
B
293
luciferase activity (light units x 10 -5)
1
3.6
2.2
2.1
28
4.2
3.6
35.5
C
COS
luciferase activity (light units x 10 -4)
1
2.13
2.3
0.8
22.7
1.5
2.9
40.06
Pit1 - + - - + + - +
Ets1 - - + - + - + +
P300 - - - + - + + +

Fig.5. p300 domain requirements. (A)Diagram of p300 domain deletion mutants. Blank spaces indicate amino acid region deleted. (B)293 cells and (C)COS-1 cells were transiently transfected with the –255PRL-Luc reporter gene and p300 mutant activity determined. The activity of each p300 mutant was assessed by transiently transfecting Pit-1/Ets-1 and each p300 species. The amount of reporter gene expression was measured as luciferase light units +/- S.E.M. normalized to an internal CMV-βgal control expression plasmid and expressed as Relative Light Units(RLU). Lysates from 293 cells were assayed for p300 protein expression by anti-FLAG western blotting(B) and Lysates from COS-1 cell transfections were immunoprecipitated with anti-FLAG antibodies then western blotted with anti-p300 antiserum(C).

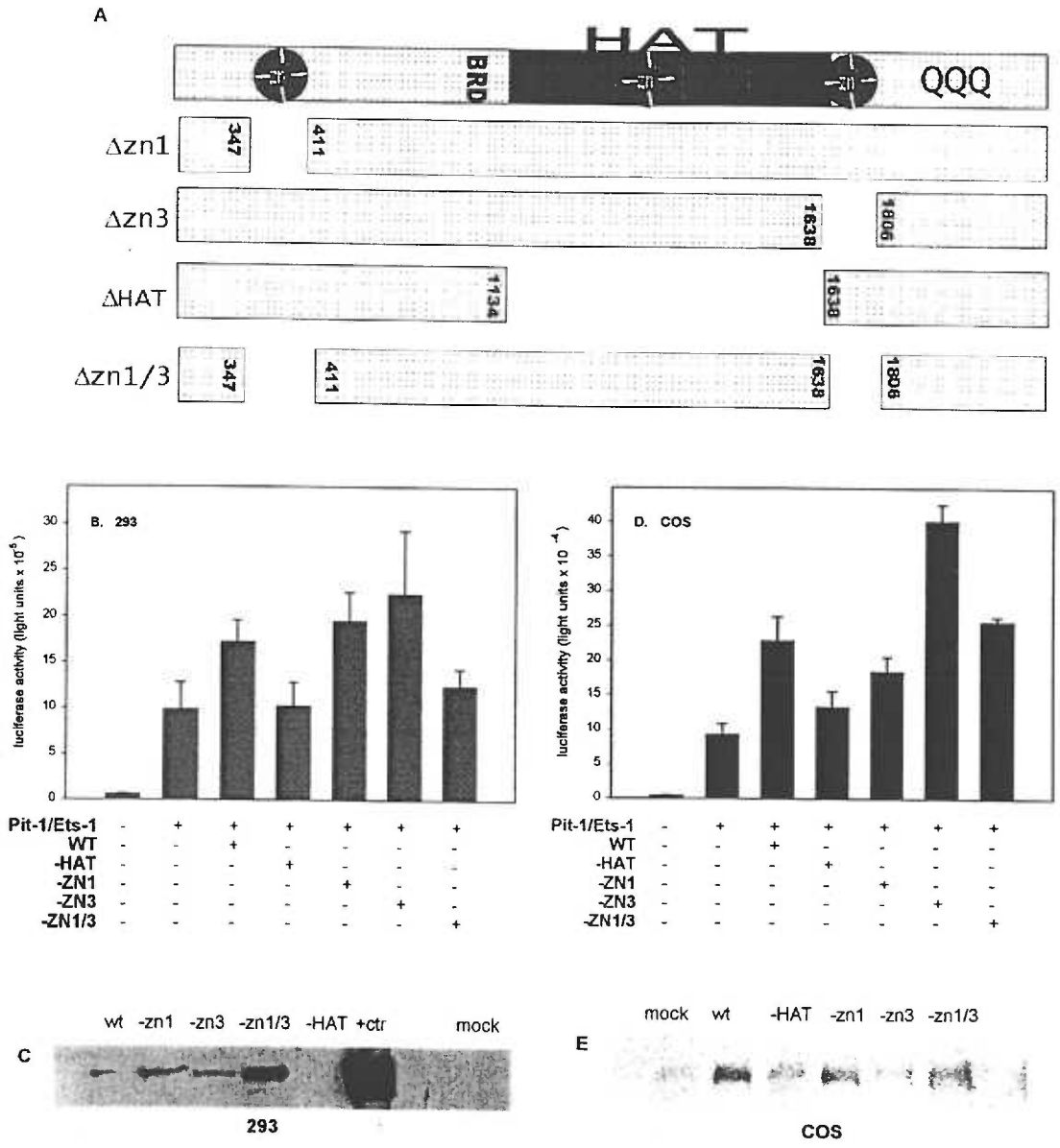
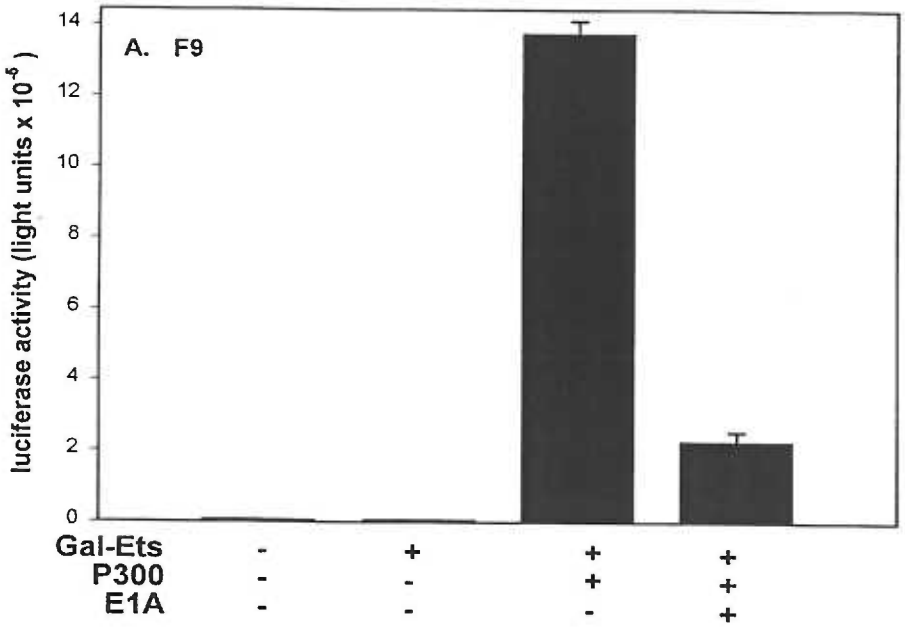

A
HAT
BRD
QQQ
Δzn1
347
411
Δzn3
1638
1806
ΔHAT
1134
1638
Δzn1/3
347
411
1638
1806
B. 293
luciferase activity (light units x 10^-5)
Pit-1/Ets-1
WT
-HAT
-ZN1
-ZN3
-ZN1/3
D. COS
luciferase activity (light units x 10^-4)
Pit-1/Ets-1
WT
-HAT
-ZN1
-ZN3
-ZN1/3
C
wt -zn1 -zn3 -zn1/3 -HAT +ctr mock
293
E
mock wt -HAT -zn1 -zn3 -zn1/3
COS

Fig.6. The adenovirus E1A protein specifically blocks p300 dependent gene expression. (a)F9 cells were transiently transfected with the 5xGal4-luc reporter along with Gal4-Ets-1, p300, and E1A expression plasmids as indicated. (b)COS-1 cells were transfected with the 5xGal4-luc reporter along with either Gal4-Pit-1 or Gal-4-Ets-1 and p300 and E1A as indicated. The amount of reporter gene expression was measured as luciferase light units +/- S.E.M. normalized to an internal CMV–βgal control expression plasmid.


A. F9
luciferase activity (light units x 10^{-5})
14
12
10
8
6
4
2
0
Gal-Ets - + + +
P300 - - + +
E1A - - - +

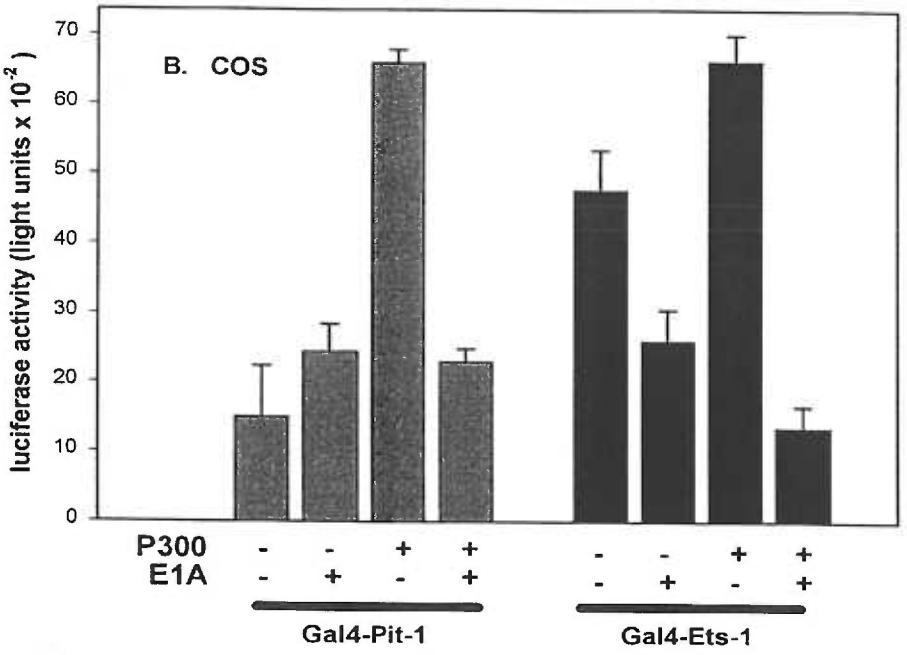

B. COS
luciferase activity (light units x 10^{-2})
70
60
50
40
30
20
10
0
P300 - - + + - - + +
E1A - + - + - + - +
Gal4-Pit-1
Gal4-Ets-1

Figure 7. The adenovirus 12S E1A protein specifically blocks Pit-1/Ets-1 dependent gene expression. COS-1 cells were transiently transfected with the −255PRL-luc reporter along with a combination of Pit-1 and Ets-1 and decreasing amounts of wild type E1A or Mutant E1A as indicated. The amount of E1A tested in this experiment ranged from 0.8ng-2.5ug. Transfection with 20ng (bars with asterisks) of wt E1A allowed for substantial inhibition of Pit-1/Ets-1 induced gene expression while transfection of the same amount of E1A mutant did not inhibit Pit-1/Ets-1 activity. Therefore, 20ng of E1A was used in subsequent assays. The amount of reporter gene expression was measured as luciferase light units +/- S.E.M. normalized to an internal CMV-βgal control expression plasmid.

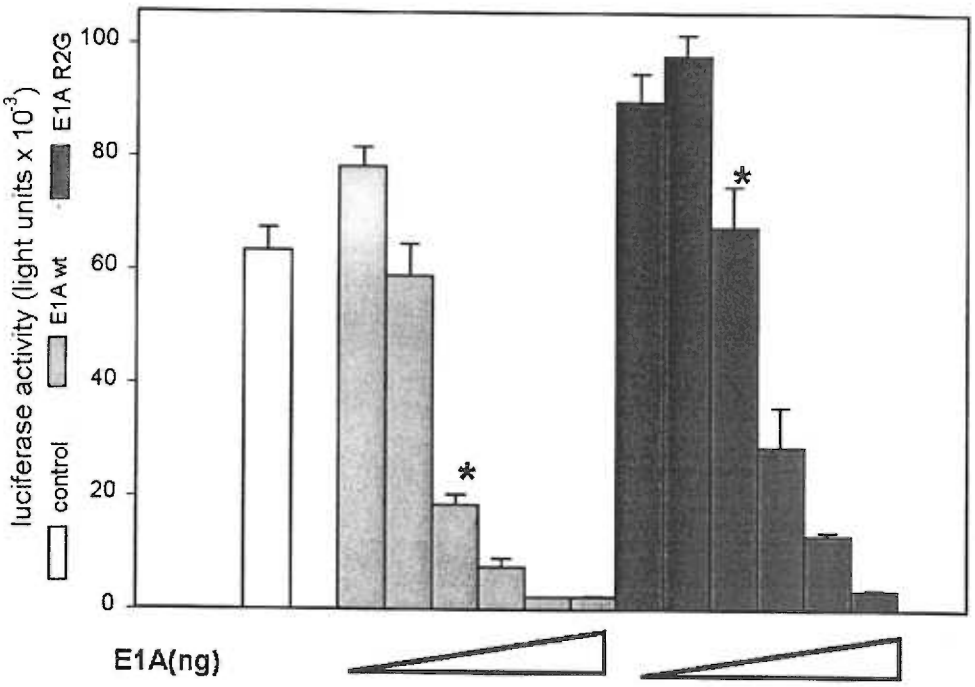

luciferase activity (light units x 10^{-3})
control
E1A wt
E1A R2G
100
80
60
40
20
0
*
*
E1A(ng)

Fig 8. Both Forskolin and EGF activation of the PRL gene is inhibited by E1A. GH3 cells were transiently transfected with the –255PRL-luc reporter and an E1A expression plasmid as indicated. Cells were then treated with FSK, GEF, or nothing as indicated. The amount of reporter gene expression was measured as luciferase light units +/- S.E.M. normalized to an internal CMV–β galactosidase control expression plasmid

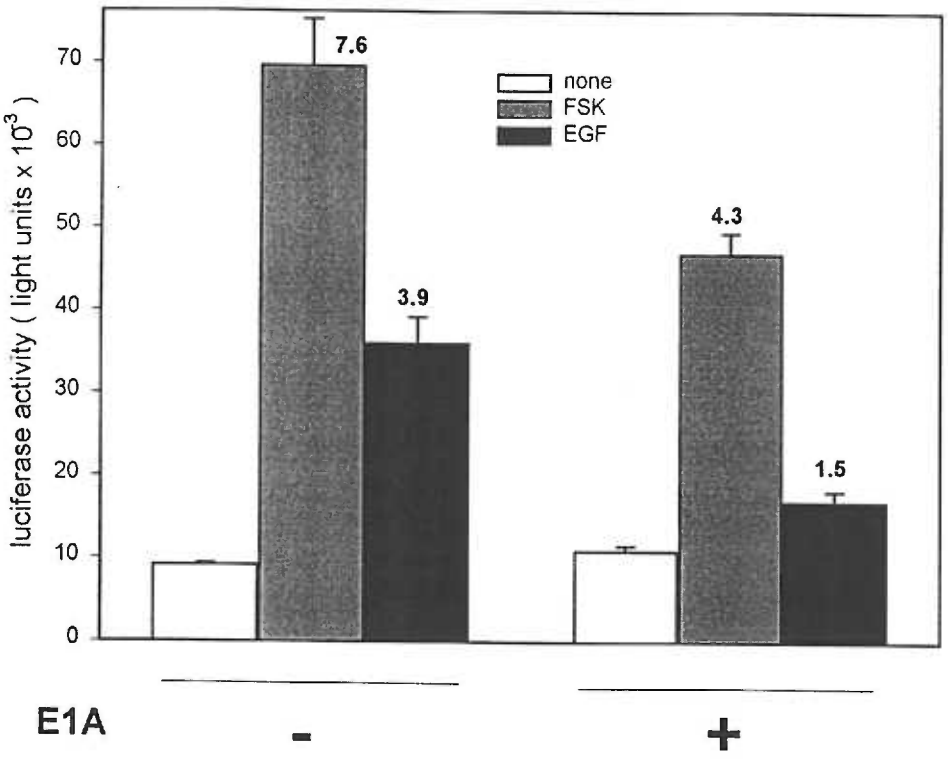

luciferase activity (light units x 10^{-3})
70
60
50
40
30
20
10
0
7.6
3.9
4.3
1.5
none
FSK
EGF
E1A
-
+

Fig 9. Both PKA and V12Ras activation of the PRL gene is inhibited by E1A. GH3 cells were transiently transfected with the –255PRL-luc reporter, an E1A expression plasmid, and PKA or V12 Ras expression plasmids as indicated. The amount of reporter gene expression was measured as luciferase light units +/- S.E.M. normalized to an internal CMV–β galactosidase control expression plasmid.

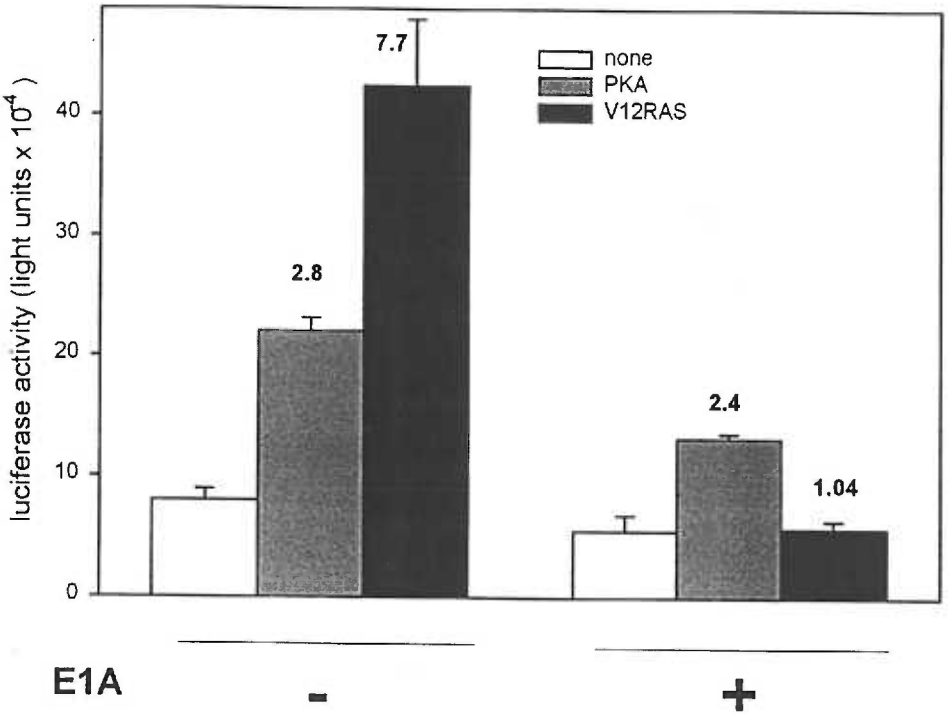
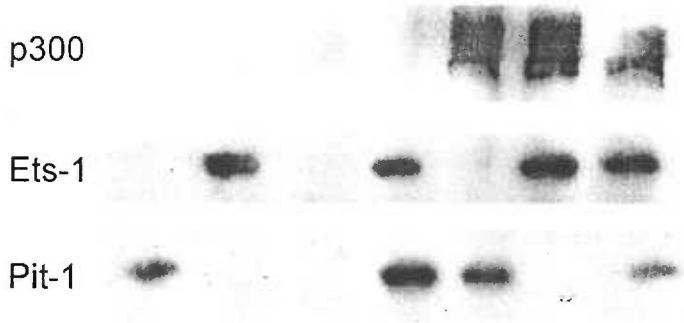

luciferase activity (light units x 10^{-4})
40
30
20
10
0
7.7
2.8
2.4
1.04
none
PKA
V12RAS
E1A
-
+

Fig.10. p300 binds to a –255PRL DNA element in a Pit-1 and/or Ets-1 dependent manner in vitro. A biotinylated–255PRL DNA element was generated via PCR and incubated, as indicated, with combinations of recombinant Pit-1, Ets-1, or p300 isolated from sf9 cells infected with bacculovirus expressing each protein. The DNA-protein complex was then incubated with streptavidin conjugated beads. DNA bound proteins were separated magnetically and subjected to SDS-PAGE, transferred to membrane and western blotted using anti Pit-1, Ets-1 or p300 antibodies.

Pit-1	+			+	+		+
Ets-1		+		+		+	+
p300			+		+	+	+



DISCUSSION

These studies provide evidence for the involvement of CBP/p300 in activation of the prolactin gene. Expression vectors for p300 were able to modestly enhance Pit-1/Ets-1 dependent expression of the proximal prolactin promoter in several heterologous cell lines. The Adenovirus E1A protein was able to substantially reduce the ability of Pit-1 and/or Ets-1 to activate expression of the prolactin promoter in COS-1 cells. The use of a specific E1A mutant expression vector suggested that the inhibition by E1A occurred through a CBP/p300 dependent mechanism. E1A was also able to completely inhibit EGF- and Ras- induced but only partially inhibit FSK- and PKA- induced activation of the prolactin gene in GH3 pituitary cells, thus implicating a differential requirement for CBP/p300 for different signaling pathways. These experiments confirm and extend the findings of previous reports which implicate CBP/p300 in the regulation of the prolactin gene.

The experimental methods employed in this report did not produce a considerable gain of function by the transfection of exogenous p300 into the tested cell lines. This was not entirely unexpected, since previous reports have observed only 3 to 4-fold activation by CBP (172, 146) or 2-fold activation by p300 (146) in reporter gene assays using CBP/p300 and CREB. It is possible that a substantial gain of function is not observed due to the presence of endogenous CBP/p300 in the cell types tested. If sufficient amounts of endogenous CBP/p300 is present at the PRL promoter, over-expression of additional p300 may not activate expression beyond the normal rate. However, this is contradictory to several reports which propose that competition for a limiting supply of CBP/p300 is the mechanism by which antagonistic signaling pathways can exert opposite effects while utilizing a common co-activator molecule (157-160). On

the other hand, our observation that E1A can effectively block Pit-1/Ets-1 induced activation of the prolactin promoter in COS-1 cells suggests that much of the ability of Pit-1 and Ets-1 to stimulate prolactin gene expression is dependent on effective levels of CBP/p300.

One possible method to discriminate between the above two opposing theories would be to use CBP/p300 deficient cell lines for transfections. As CBP/p300 deficient tissue culture strains are not available, experimental methods which artificially diminish CBP/p300 gene expression or deplete cells of CBP/p300 protein might allow the exogenous introduction of p300 to exhibit a clear gain of function phenotype. Several methods might be employed to accomplish this task. For example, introduction of antisense oligonucleotides into tissue culture cells could reduce CBP/p300 translation to low enough levels that might allow for the assessment of whether CBP/p300 is required for Pit-1/Ets-1 dependent PRL gene expression. Subsequent over-expression of CBP/p300 may then allow for the attainment of gain of function phenotypes, provided that the expression levels of the introduced CBP/p300 are high enough to overcome inhibition by the antisense oligonucleotides. This may be accomplished by the introduction of a "pseudo wild type" p300 which is not recognized by the antisense oligonucleotide. Also, co-microinjection of specific antibodies for p300 along with p300 expression vectors encoding full length p300 or p300 deletions into tissue culture cells might be a viable technique to determine if specific domains of p300 are required for the expression of the prolactin gene. In this case, optimization of the balance between antibody and expression vector must be attained if the introduced protein is not to be affected by the specific antibodies. A pseudo wild type p300 expression vector might also be useful in this type of experiment.

Another possibility for the observed modest enhancement of prolactin expression by p300 is that the HAT activity of p300 is the primary function which defines its role as a co-activator for the PRL gene. Since the reporter gene constructs in the co-transfection assays may not be present in the form of "normal" chromatin, the HAT activity of p300 may not be necessary nor utilized to enhance transcriptional activation via the acetylation of histones. If modification of chromatin structure is the primary function of CBP/p300 as a co-activator protein, then the activity of p300 and the mutant p300 constructs could be tested in in vitro transcription assays (145) using -255PRL chromatin templates. Purified mutant p300 proteins would be added to –255PRL templates which were assembled into chromatin in the presence of Pit-1 and/or Ets-1 and the relative amounts of transcription would be determined by primer extension analysis. This assay would also provide evidence for CBP/p300's dependence on either Pit-1 or Ets-1 for transcriptional activation.

Yet another possible explanation for the low level of p300-induced enhancement of Pit-1/Ets-1 dependent PRL expression may lie with the use of p300 itself. Although CBP and p300 are considered functional homologues, CBP and p300 have been shown to exhibit distinct roles in certain biological systems. For instance, haploinsufficiency of one allele of CBP can lead to severe defects in humans, resulting in Rubenstein-Taybi syndrome (161) and in mice (162). These individuals are apparently wild type for p300 and p300 does not appear to be able to compensate for the lack of the CBP allele. Additionally, a recent report by Kawasaki et al (163) used specific hammerhead ribozymes for CBP or p300 to demonstrate distinct roles for CBP and p300 in the retinoic acid induced differentiation of F9 cells. Therefore, if CBP was used in our experiments instead of p300, we might have obtained a different level of PRL gene expression. This theory could be easily tested by substituting CBP expression vectors,

including the homologous deletion mutations, for the p300 expression vectors used in our assays.

If p300 is capable of enhancing the Pit-1/ Ets-1 dependent expression of the prolactin gene, one might expect to see an increased affinity of p300 for a Pit-1/Ets-1/DNA complex when compared to a Pit-1/DNA or Ets-1/DNA complex. Surprisingly, this was not observed in our DNA-avidin-biotin-complex assays. p300 did not appear to preferentially bind to the PRL promoter with both Pit-1 and Ets-1 vs. with either transactivating factor alone. This doesn't preclude the possibility that p300 becomes preferentially activated by the simultaneous contact with both Pit-1 and Ets-1. A possible explanation for the apparent lack of enhanced p300 binding to the PRL promoter element in the presence of Pit-1 and Ets-1 may lie in the conformation of the Pit-1 and Ets-1 bound prolactin promoter element. It is possible that the recombinant proteins were present in this assay in such a large excess that low affinity binding occurred between the Pit-1 and Ets-1 and the promoter element. This may have distorted the native binding conformation of Pit-1 and/or Ets-1 and as a result, the Pit-1 and Ets-1 binding sites normally encountered by p300 may no longer be in the optimal p300 binding configuration. This possibility could be tested with further optimization of the concentrations of each component of this assay. Additionally, in vitro footprinting analysis may provide information about the binding configuration of Pit-1 and Ets-1 to the proximal prolactin promoter element in this assay. An alternative method may be found with an in vivo chromatin immunoprecipitation assay (145, 164, 165). This method should allow for the verification of the recruitment of p300 to the prolactin promoter if specific antibodies to CBP/p300 were used to immunoprecipitate formaldehyde crosslinked chromatin-protein complexes. The prolactin promoter could

then be detected by southern blotting or PCR analysis if it immunoprecipitated along with CBP/p300.

While EGF, V12Ras, FSK, and PKA activation of the –255PRL-luciferase reporter were all inhibited by E1A, differences in the inhibition patterns were seen. E1A was able to inhibit EGF and V12Ras induced activation to nearly basal levels, while it appeared to only partially inhibit FSK and PKA induced reporter gene activity. This observation suggests that an E1A sensitive, CBP/p300 activity is involved in the EGF/Ras and FSK/PKA induced activation of the prolactin gene. But the differential inhibition by E1A suggests a differential requirement for a CBP/p300 activity in mediating responses to EGF/Ras vs. FSK/PKA. The near complete inhibition of EGF/V12Ras induced activation of the prolactin gene by E1A suggests that the EGF/Ras activation pathway is dependent on a CBP/p300 activity. The partial insensitivity to E1A by FSK/PKA activation suggests that there may be both a CBP/p300 independent and CBP/p300 dependent component of the FSK/PKA signaling pathway. The same CBP/p300 independent mechanism may also be involved in basal expression of the prolactin gene as E1A had no inhibitory effect on basal expression of the –255PRL reporter gene.

One mechanism by which E1A is thought to function to disrupt normal cellular processes is by displacing transcription factors and co-activators from CBP/p300 (116, 166-170). E1A binds to the 3rd zinc finger (C/H3) region of CBP/p300 (171) which is also recognized by a number transcriptional activators including TFIIB (172),TBP (173), RNA helicase A (174), and P/CAF (175). It is possible that the E1A inhibition seen in the previous experiments is due to the displacement of one of these factors from CBP/p300 at the prolactin promoter. Since E1A had no effect on basal expression of the prolactin reporter, it is likely that displacement of components of the general transcription

machinery i.e. TFIIB, TBP, or RNA helicase A is not the primary mechanism by which E1A is able to inhibit expression of the prolactin gene. Disruption of the functional interaction between CBP/p300 and P/CAF is a possible mechanism for inhibition of the prolactin gene by E1A. EGF or Ras activation of the prolactin gene may require the recruitment of P/CAF to the promoter. FSK or PKA activation of the prolactin gene may signal through two divergent pathways, one which requires the recruitment of P/CAF, while the other may not. This idea of differential co-activator requirements is supported by a recent report from Xu et al (134). Xu showed that different HAT activities from different proteins are required by a Pit-1 response element when activated by cAMP vs. growth factors. The HAT function of CBP was required to reverse the inhibition of Pit-1 activity by the injection of anti-CBP antibodies into 293 cells when activated by forskolin. The HAT function of P/CAF was required to reverse the inhibition of Pit-1 activity by the injection of anti-P/CAF antibodies into 293 cells when activated by insulin. This demonstrated that Pit-1 activity had a different requirement for co-activator domains when 293 cells were activated by cAMP vs. growth factors. Our data fits this model such that a P/CAF activity may be required for activation of the prolactin proximal promoter by EGF/Ras. FSK/PKA activation of the prolactin gene could then be accomplished by two different signaling pathways, one of which may require the P/CAF activity while the other does not. Recent studies have demonstrated that P/CAF can bind to several transcription factors such as RAR (203), the human cytomegalovirus factor IE86 (201), and a cyclin D1/estrogen receptor complex (202) independently of CBP/p300. Thus, it is also possible that activation of the prolactin promoter by P/CAF may be independent of a CBP/p300 activity. Although the interaction of P/CAF with either Pit-1 or Ets-1 remains to be tested, it is possible that the CBP/p300 independent component of the FSK/PKA activation pathway could involve the recruitment of P/CAF directly to Pit-1

and/or Ets-1 at the prolactin promoter. The interaction between P/CAF and Pit-1 or Ets-1 could be investigated using in vitro GST-fusion pulldown experiments and in vivo co-immunoprecipitation experiments. Future studies which more closely examine the role of P/CAF in the regulated expression of the prolactin gene will certainly shed more light on the ideas proposed in this report.

The question of whether a CBP/p300 independent pathway of FSK/PKA activation exists may possibly be addressed by utilizing specific CBP/p300 catalytic ribozymes (163) to inhibit the expression of CBP/p300 and test whether the responses to FSK/PKA or EGF/Ras are dependent upon the expression of CBP/p300. If, for example, the activation of the prolactin promoter by FSK/PKA is, in part, independent of p300 recruitment to the promoter, then cells expressing the CBP and p300 specific ribozymes should still respond to treatment by FSK/PKA. This type of assay may also be helpful in determining if both or either CBP and p300 are involved in the expression of the prolactin gene since catalytic ribozymes for either CBP or p300 (163, 176) could be introduced into cell lines and block specific expression of either CBP or p300 to test for specific effects of each related co-activator on the activation of the prolactin promoter. We may also use this assay system to analyze the effects of P/CAF on expression of the PRL gene, although this would first necessitate the development of a P/CAF specific catalytic ribozyme.

CHAPTER 3

Analysis of the Role of the Mitogen Activated Protein Kinase in Mediating cAMP effects on Prolactin Promoter Activity

INTRODUCTION

Although it has been known for some time that cAMP can stimulate PRL gene expression (59, 151), it has been difficult to determine the mechanisms mediating this response. Studies utilizing expression vectors of the catalytic subunit of the cAMP-dependent protein kinase (PKA) (153) or the heat stable inhibitor of PKA (152), have provided evidence that PKA is sufficient and necessary for cAMP-induced activation of the PRL gene. However, the events downstream of PKA have been elusive. Unlike many PKA-regulated genes, stimulation of PRL gene expression by cAMP probably does not involve CREB (66, 177). Several studies have provided evidence that the pituitary-specific POU transcription factor, Pit-1, may play a role in mediating the effects of cAMP (63, 177, 178) as well as other signaling pathways (179, 180) on PRL transcription. The finding that Pit-1 is phosphorylated in response to elevated cAMP levels in GH_3 cells (181, 182) rather strongly supported the view that Pit-1 may function as a cAMP-regulated transcription factor. However, studies using Pit-1 mutants in which the phosphorylation site is removed (183, 184) have provided evidence that cAMP-induced activation of the PRL promoter probably does not require phosphorylation of Pit-1. Thus, it is by no means clear that phosphorylation of Pit-1 mediates cAMP effects

on PRL transcription and other mechanisms need to be considered. We have obtained evidence that cAMP can activate the MAPK-responsive transcription factor, Elk-1, providing indirect evidence that cAMP may stimulate MAP kinase in GH_3 cells (185) raising the possibility that the MAPK pathway may be involved in mediating effects of cAMP on transcription of the PRL gene. We have also recently obtained evidence that thyrotropin releasing hormone (TRH) effects on prolactin transcription may be mediated at least in part through activation of MAPK (111). Thus it is possible that both the TRH and cAMP signaling pathways may utilize the MAPK cascade to stimulate PRL gene expression.

In the present study we have examined the ability of cAMP to stimulate MAPK activity and tested the role that cAMP-induced MAPK activation plays in modulating PRL transcription. We have found that cAMP can activate MAPK in GH_3 cells and that the increase in MAPK is required for full effects of cAMP on PRL transcription. Analysis of DNA sequences of the prolactin gene which are required for cAMP-responsiveness reveals both similarities and differences in role of specific Ets factor binding sites for TRH- and cAMP-responsiveness. We have also assessed the possible role that the transcriptional co-activators, CREB binding protein and p300, play in mediating cAMP-induced activation of the PRL promoter.

MATERIALS and METHODS

Materials

Tissue culture reagents and media were purchased from Life Technologies (Gaithersburg, MD). Antibodies to ERK1, ERK2, phosphorylated ERK, anti-mouse and rabbit horseradish-peroxidase conjugated IgG and immunoprecipitation reagents were

acquired from Santa Cruz Biotechnology, INC (Santa Cruz, CA). The chemical inhibitor PD98059 was obtained from Alexis Corporation (San Diego, CA) and H-89 and genistein were purchased from Calbiochem (La Jolla, CA). Forskolin and chloro-phenyl-thio-cyclic-AMP (CPT-cAMP) were purchased from Sigma (St. Louis, MO). Epidermal growth factor (EGF) was from Roche Molecular Biochemicals (Indianapolis, IN). Thyroid releasing hormone (TRH) was purchased from Peninsula Laboratories, Inc. (San Carlos, CA). Radioisotopes and chemiluminescence reagents were obtained from Dupont, NEN (Boston, MA).

Reporter Genes and Expression Vectors

A prolactin reporter construct containing sequences representing the -255 to +34 region of the rat prolactin gene (186) was obtained by polymerase chain reaction and inserted upstream of the firefly luciferase coding sequence in the pLuc-Link vector (187). Individual consensus Ets factor binding sites in the PRL 5' flanking region were disrupted by oligonucleotide-directed mutagenesis using polymerase chain reaction. The specific mutations for each site were as follows: the Ets sites at –211 to –208 and -183 to –180 were mutated from TTCC to TGAA, the site at –162 to –159 from TTCC to GGCC, the site at –95 to –92 from GGAA to GTTC and the site at –75 to –72 from GGAA to GGCC.

A GAL4-p300 construct was generated by in-frame subcloning of the full coding sequence of p300 into a vector containing the GAL4 (1-147) DNA binding domain downstream of the cytomegalovirus promoter (188). The RSV-PKI vector (152), RSV-Pit-1 vector (63), GAL4-Elk (189) construct and the luciferase reporter containing 5 GAL4 binding sites (188) have been described previously. The CMV-Ets-1 expression vector was provided by Dr R. Goodman. The prolactin promoter serial deletions were graciously provided by Paul Keivit and Ying Hong Wang from the Maurer lab.

Cell Culture and Transfection

GH_3 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 2.5% fetal bovine serum and 15% equine serum. For transfection, GH_3 cells were seeded into 6-well dishes 1 day prior to transfection at an approximate density of 500,000 cells per well. DNA (1-3 μg total) was transfected using the Lipofectamine reagent (Life Technologies) according to the manufacturer's protocol. After overnight incubation, the cells were treated with agonists for 6 hours after which the cells were washed, lysed, and analyzed for luciferase activity. All experiments with the PD98059 compound were performed in DMEM supplemented with BSA (6.6 mg/ml) to prevent precipitation of PD98059. Cells were transfected with CMV-β-Galactosidase expression vector as an internal standard (190).

Immunoblot analysis

For immunoblot analysis, GH_3 cells were grown to ~80% confluency, cultured in serum-free medium for 24 hours, and treated with agonist for specific times. Cells were lysed in BOS buffer (50 mM Tris-HCl, pH8.0, 10% Glycerol, 1% Nonidet-P40, 200 mM NaCl, 2.5 mM $MgCl_2$, 2 mM sodium vanadate) supplemented with a mixture of protease inhibitors (Complete Proteinase Inhibitor, Roche Molecular Biochemicals, Indianapolis, IN). Equal amounts of protein (50 μg) were loaded on a 10% denaturing polyacrylamide gel. Proteins were transferred to a polyvinylidene difluoride (PVDF; Millipore Co, Bedford, MA) membrane before incubation with antibodies to detect specific proteins. All antibodies were used at a concentration of 1:1000 in TBS-T, 5% non-fat milk. The proteins were visualized on radiography film using a secondary antibody conjugated to horseradish peroxidase and a chemiluminescence detection kit (Dupont NEN, Boston, MA).

RESULTS

Elevation of cAMP induces MAPK activity in GH_3 cells.

Previous studies from this laboratory used an indirect assay involving transcriptional activation of a GAL4-Elk1 fusion protein to test for cAMP effects on MAPK activation (132). To directly assess MAPK activation, we used antibodies that detect phosphorylation of ERK1 and ERK2 on the activation loop. ERK1 and ERK2 are activated by phosphorylation of specific tyrosine and threonine residues (191) which can be detected using appropriate antibodies. Treatment of cells with forskolin resulted in the rapid stimulation of the phosphorylation of ERK1 and ERK2 (Fig 1). There was little or no change in the level of total immunoreactive ERK1 and ERK2 (data not shown) suggesting that the increased phosphorylation is not due to a change in the total amount of these kinases. This finding confirms our earlier results (132) and studies from Jacob and Stanley (192) which demonstrated cAMP-induced activation of MAPK in the GH4 cell line. The present study also provides information about the time course of MAPK activation by cAMP.

MAPK activation is required for cAMP-stimulated transcriptional activity

To determine if cAMP-stimulated MAPK activity is required for specific gene expression, we used the inhibitor, PD98059. PD98059 interacts with MEK1 with a high affinity for MEK1 and can block the ability of MEK1 to activate ERK1 and ERK2 (193). GH_3 cells were pretreated with 100 μM PD98059 for 30 minutes and then treated with cAMP for 5 minutes and assayed for ERK phosphorylation (FIG.2). PD98059 treatment greatly reduced cAMP-stimulated phosphorylation of ERK1 and ERK2. The ability to PD98059 to block cAMP induced transcriptional activation was then tested (Fig. 2B and 2C). PD98059 strongly reduced the ability of forskolin to stimulate GAL4-Elk1 activity as

assessed by a GAL4-dependent reporter gene (Fig. 2B). This finding is consistent with our observation that cAMP induces MAPK activity and the known ability of MAPK to induce Elk1 activity (194). PD98059 reduced, but did not completely block forskolin effects to induce a PRL reporter gene (Fig. 2C). Similar results were obtained when a kinase-defective MEK1 was used to inhibit activation of the MAPK pathway (data not shown). These findings provide evidence that cAMP-induced activation of MAPK plays a key role in stimulating Elk1 transcriptional activation and also contributes to cAMP-induced enhancement of PRL transcription.

Analysis of the cAMP Responsiveness of 5' Prolactin Promoter Deletion Mutants

To identify cAMP responsive cis-elements within the proximal prolactin promoter, a series of 5' deletion PRL-luciferase reporter constructs were prepared. The 5' deletion mutants were transfected into GH3 cells. The cells were then treated with cpt-cAMP and the luciferase activity of each mutant was measured (fig. 3). Contrary to the findings of others (178)(195) which identified a discrete cAMP responsive cis element between –101 and –92, we observed a graded loss of cAMP responsiveness for each successive deletion mutant. Thus, no single region of the proximal prolactin promoter appears to be responsible for conveying the response to cAMP.

Analysis of the role of ETS binding site in mediating cAMP-induction of the prolactin promoter

The proximal region of the prolactin gene contains a number of consensus sites for members of the Ets family of transcription factors (Fig. 4A). As MAPK has been shown to stimulate the transcriptional activity of several members of the Ets family, (194, 196), the Ets sites appear to be excellent candidates for mediating the transcriptional response to cAMP-induced MAPK activation. Indeed, previous studies have shown that disruption of some of the Ets sites can reduce transcriptional responses to Ras (113) or

TRH (111). To determine if any of the Ets sites play a role in cAMP-dependent prolactin gene expression, prolactin reporter constructs were tested for cAMP, TRH and EGF responsiveness (Fig. 4B). Some of the Ets site mutants were previously described (111) and several Ets site mutants were newly prepared for this study. The only mutation which reduced cAMP, TRH and EGF responsiveness to less than 50% of wild type was mutation of the Ets site located at position –211, a site which previously has been shown to be important for MAPK responsiveness (111, 113). Disruption of other Ets sites either only modestly reduced responsiveness, or in some cases stimulated reporter gene activation in response to cAMP, TRH or EGF.

As there are multiple Ets sites in the proximal region of the PRL gene, it seemed possible that modest the effects of mutating single Ets sites reflected some redundancy in their ability to confer MAPK responsiveness. To assess the overall contribution of Ets sites within the proximal region of the prolactin gene, a reporter construct was created in which all the consensus Ets sites were mutated. This construct was tested for its ability to be activated by the activators forskolin, EGF and TRH (Fig. 4C). Surprisingly the construct in which all of the Ets sites were mutated was more responsive to forskolin treatment than the wild type PRL promoter. In contrast, and as expected, reporter gene responses to TRH and EGF were reduced in the Ets mutant. Thus although cAMP, TRH and EGF all lead to activation of MAPK, the requirements for downstream transcription factor targets of these signaling pathways appear to differ.

Elevation of cAMP enhances p300 transcriptional activity in a MAPK-dependent manner

As the preceding studies suggest that Ets factors are probably not required for transcriptional responses to cAMP, we considered a possible role for other factors. Recent studies have provided evidence that the ability of cAMP to stimulate PRL

promoter activity in heterologous is dependent on the closely related co-activator proteins, CBP and p300 (134, 197). To test the role of endogenous CBP and/or p300 in mediating responsiveness of the PRL promoter to cAMP, we used an expression vector for the adenovirus 12S E1A protein, which blocks CPB and p300 activity (146, 147). We found that the E1a expression vector reduced the ability of cAMP to stimulate PRL promoter activity in GH_3 cells (See chapter 2, figure 9. this thesis). This finding offers further support for the view that CBP/p300 plays a role in transcriptional regulation of the PRL gene. To further explore this topic, we elected to test the ability of cAMP to stimulate the transcriptional activity of p300. We prepared an expression vector for a GAL4-p300 fusion which was co-transfected with GAL4-dependent reporter gene. The results demonstrate that GAL4-p300 activity as substantially increased by treatment with forskolin (Fig. 5). Interestingly, treatment with the MEK inhibitor, PD98059, almost completely blocked the ability of forskolin to enhance GAL4-p300 activity. These findings offer evidence that in GH_3 cells, cAMP stimulates p300 activity in an MAPK-dependent manner.

Fig. 1. Time Course of cAMP-Mediated MAPK Activation

immunoblot analysis of ERK activation. GH_3 cells were cultured in serum-free medium for 24 hours and treated with 10 μM forskolin for the indicated times. Cell lysates (50 μg) were resolved on a 10% denaturing polyacrylamide gel, transferred to a membrane, and phosphorylated ERK1 and ERK2 were detected with a phosphorylation-specific antibody.

Forskolin Treatment (minutes)						
0	2	5	10	15	25	40

Fig. 2. MAPK Activation is Required for Full Effects of cAMP on prolactin Gene Expression

Analysis of the effects of the MAPK inhibitor, PD98059, on forskolin-induced ERK activity in GH_3 cells (A). GH_3 cells were cultured in serum free medium for 24 hours, pretreated for 30 minutes with vehicle (DMSO) or PD98059 (100 μM) followed by forskolin treatment (10 μM) for 5 minutes. Cell lysates were resolved on a 10% poly-acrylamide denaturing gel, transferred to a membrane, and probed with an antibody specific to the phosphorylated form of ERK (A). Analysis of the effects of the MAPK inhibitor, PD98059 on forskolin-induced reporter gene activity (B and C). GH_3 cells were transfected with either a GAL4-dependent luciferase reporter gene and an expression vector encoding a GAL4-Elk1 fusion protein (B) or a prolactin promoter containing the proximal 255 base pairs fused to the luciferase reporter gene (C). At 24 hours after transfection, GH_3 cells were pretreated for 30 minutes with either the vehicle (DMSO) or the inhibitor PD98059 (100 μM). The agonists forskolin (10 μM) and EGF (10 nM) were added for 6 hours after which the cells were lysed and analyzed for luciferase activity. Reporter gene activity is reported as light units from three independent transfections ± S.E.M. normalized to β-galactosidase activity

Pretreatment	Control		PD98059	
Forskolin	-	+	-	+

A. Phospho-ERK

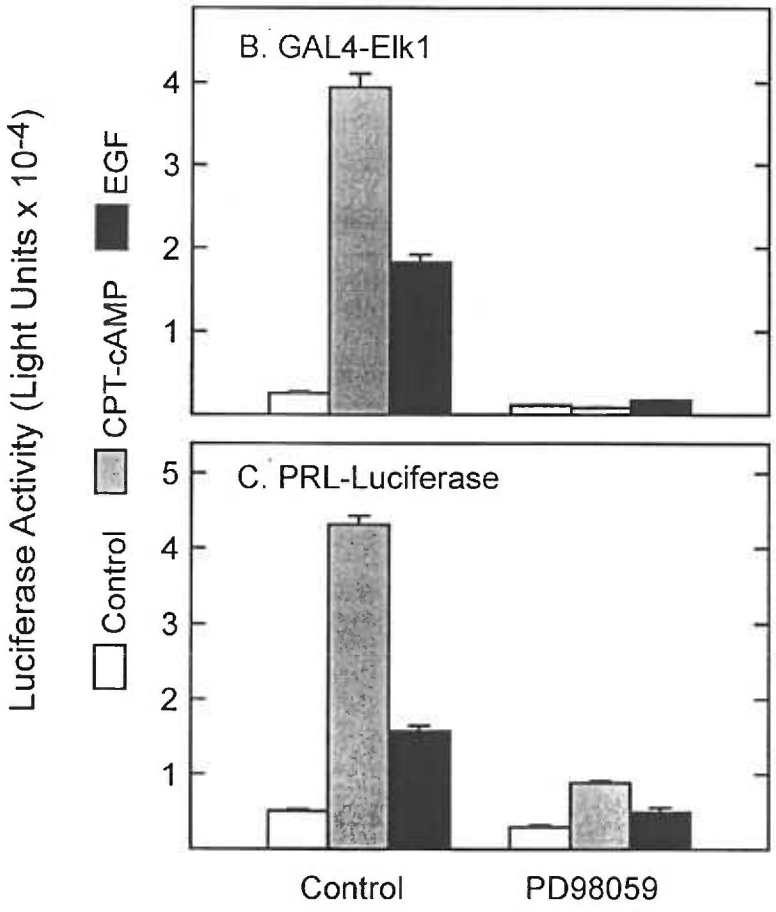


Fig. 3. Analysis of the cAMP Responsiveness of 5' Prolactin Promoter Deletion Mutants.

5' deletion prolactin promoter mutants were transiently transfected into GH3 cells and treated with cpt-cAMP as described in methods. Reporter gene expression was measured as relative luciferase light units and expressed as % induction relative to untreated control. Error bars represent +/- S.E.M. normalized to internal β-galactosidase activity.

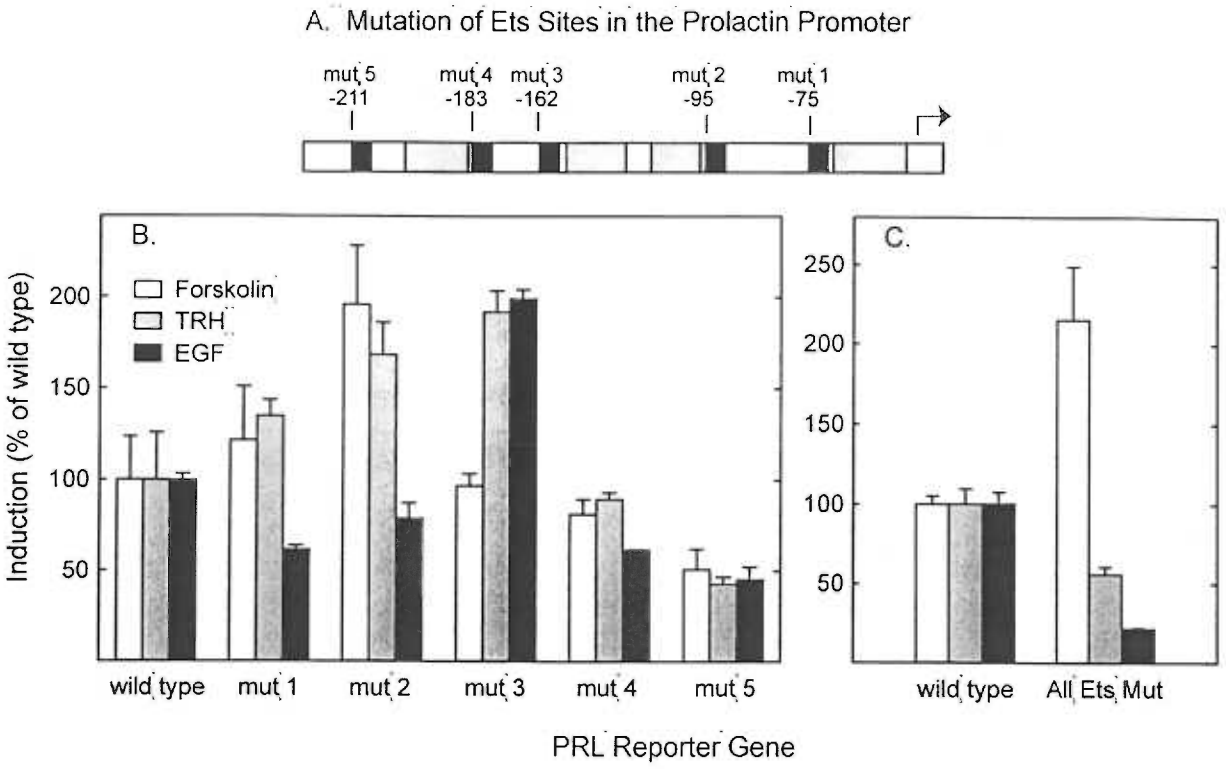

A. Mutation of Ets Sites in the Prolactin Promoter
mut 5
-211
mut 4
-183
mut 3
-162
mut 2
-95
mut 1
-75
B.
Forskolin
TRH
EGF
Induction (% of wild type)
200
150
100
50
wild type
mut 1
mut 2
mut 3
mut 4
mut 5
C.
250
200
150
100
50
wild type
All Ets Mut
PRL Reporter Gene

Fig. 4. Mutational Analysis of the Putative Ets Binding Sites in the Proximal Prolactin Promoter

Specific Ets factor binding sites within the proximal region of the prolactin gene were disrupted as indicated by the schematic diagram for the prolactin promoter (A). GH_3 cells were transfected with prolactin promoter reporter constructs in which individual Ets factor binding sites was disrupted (B) or a reporter gene in which all of the consensus Ets sites were disrupted (C). After serum deprivation, the cells were treated with forskolin (10 μM), TRH (100 nM) or EGF (10 nM) for 6 hours. Cells were collected and assayed for luciferase activity. Reporter gene activity is reported as the light units three independent transfections ± S.E.M. normalized to β-galactosidase activity.

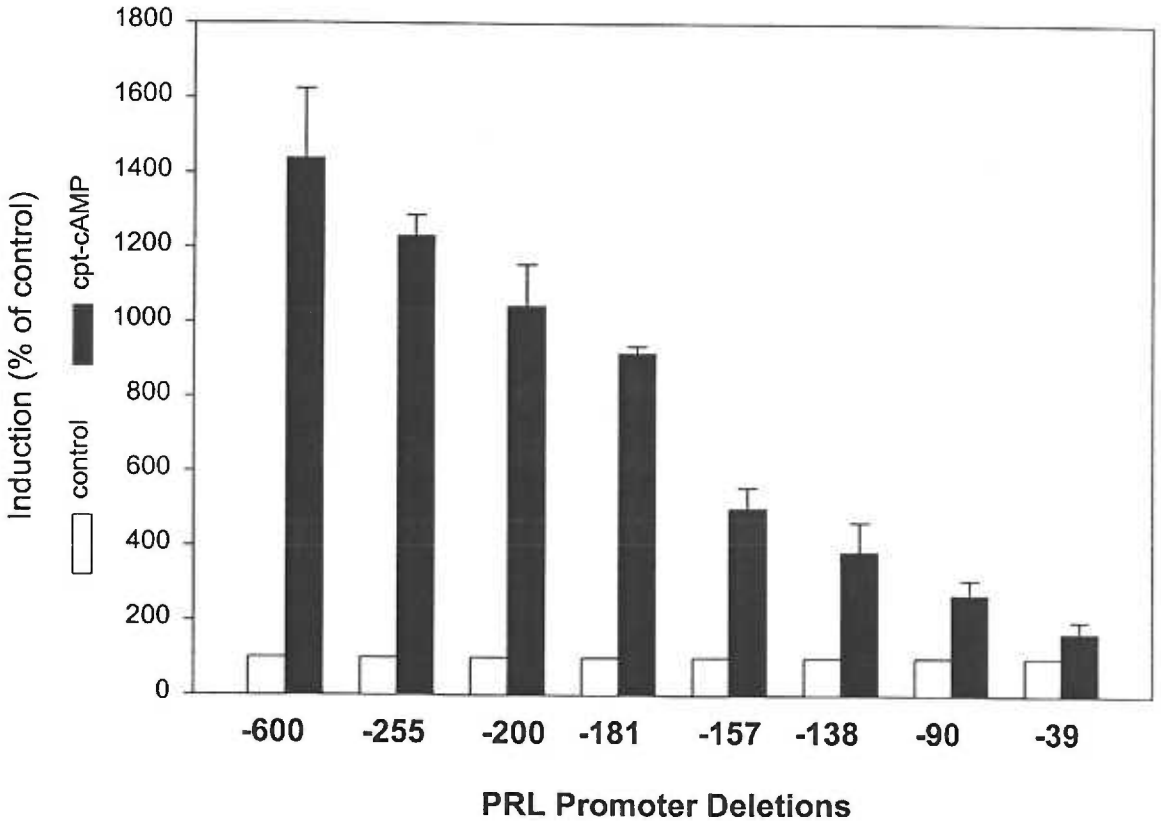
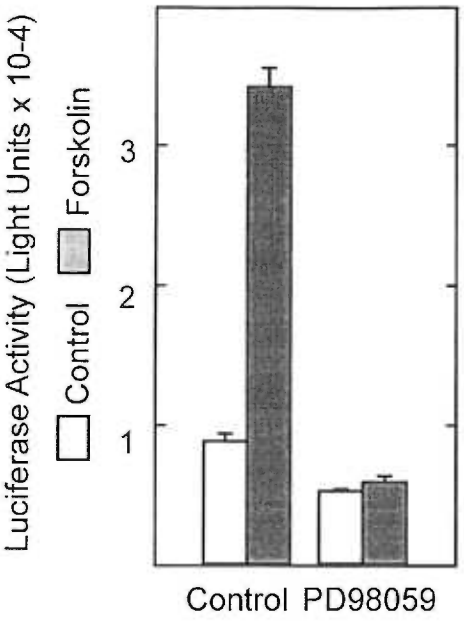

Induction (% of control)
1800
1600
1400
1200
1000
800
600
400
200
0
control
cpt-cAMP
-600
-255
-200
-181
-157
-138
-90
-39
PRL Promoter Deletions

Fig. 5. Elevation of cAMP levels can stimulate activity of a Gal4-p300 fusion protein.

Analysis of PD98059 effects on forskolin-induced activation of a GAL4-p300 fusion protein. GH_3 cells were transfected with an expression vector encoding a GAL4-p300 fusion protein and a reporter construct containing 5 GAL4 binding sites. The cells were serum deprived for 24 hours and treated with the inhibitor PD98059 for 30 minutes. Forskolin (10 μM) was added and cells were collected and assayed 6 hours later. Reporter gene activity is reported as light units from three independent transfections ± S.E.M. normalized to β-galactosidase activity.


Luciferase Activity (Light Units x 10-4)
Control
Forskolin
3
2
1
Control
PD98059

Discussion

These studies provide evidence that cAMP can activate the MAPK signaling pathway in GH_3 cells and that MAPK activation contributes to the ability of cAMP to stimulate PRL promoter activity. Analysis of ERK phosphorylation as well as immunocomplex assay (unpublished observation by Paul Kievit in the Maurer lab) of ERK activity have provided strong evidence that elevated cAMP can stimulate MAPK activity. Inhibition of the MAPK pathway was found to reduce the ability of cAMP to activate the PRL promoter and a GAL4-Elk1 fusion protein. These findings as well as previous studies from this lab (111) provide that the cAMP, EGF and TRH signaling pathways all converge in GH_3 cells to stimulate MAPK activity which then plays a role in stimulating PRL gene expression. Unpublished observations by Paul Kievit in the Maurer lab suggest that the small GTP binding protein Rap1 may be partially responsible for mediating the cAMP-induced MAPK activation of the prolactin promoter in GH3 cells.

Concerning the events downstream of MAPK, it seemed likely that members of the Ets family of transcription factors would be involved in mediating transcriptional regulation of the PRL gene. Previous studies have led to a model in which Ras, TRH or EGF can stimulate MAPK activity leading to Ets factor phosphorylation and transcriptional activation involving several specific DNA elements in the PRL gene (110, 111, 113, 132, 143). As the present studies demonstrate that cAMP can stimulate MAPK activity, it seemed probable that Ets sites would also play a role in mediating transcriptional responses to cAMP. To test this possibility, Ets binding sites within the proximal region of the PRL promoter were mutated, including several Ets sites which have not previously been studied. Similar to previous studies examining Ras- or TRH-

responsiveness (111, 143), mutation of the Ets site at position –211, decreased cAMP-responsiveness to less than 50% of wild type activity. Interestingly, this region of the PRL gene has been shown to interact with the LIM homeodomain transcription factor, Lhx3 (198), and Lhx3 has been shown to enhance Ras-responsiveness of the prolactin promoter (182). Mutation of other single Ets sites either had smaller effects, or actually stimulated cAMP-responsiveness. The increases in responsiveness which were observed at some sites may be due to disruption of the binding of a repressor. It has been reported that some Ets factors can function as repressors and one has been shown to inhibit PRL promoter activity (199, 200). To further explore the overall role of Ets sites, a PRL promoter reporter gene construct in which all of the consensus Ets were disrupted was prepared. Surprisingly, the promoter construct with all Ets sites disrupted was even more responsive to cAMP than the wild type reporter gene. In contrast, disruption of all of the consensus Ets sites substantially reduced EGF-responsiveness and partially reduced TRH-responsiveness. Thus, it appears that there is a differential requirement for Ets sites in mediating regulation of the PRL promoter. The consensus Ets sites are not required for cAMP-responsiveness, but are necessary for full responses to EGF or TRH. However, the possibility exists that Ets factors may still be recruited to the proximal prolactin promoter via contact with Pit-1 or other factors present at the promoter irrespective of actual DNA binding by the Ets factor. This possibility may be tested through several methods. The use of a chromatin immunoprecipitation assay with transiently transfected wild type or all Ets mutant prolactin promoters and specific antibodies against Ets-1 may allow the presence of Ets-1 at the mutant promoter to be determined, although the chromatin structure of the prolactin templates may not be 'normal'. Also, an in vitro electrophoretic mobility shift assay might be performed with either the wild type or mutant prolactin promoter element

and nuclear extracts from pituitary cell lines. The presence of Ets-1 at the mutant promoter could then be assessed by 'super-shifting' with specific Ets-1 antibodies.

The ability of the PRL promoter to respond to cAMP appears to involve the transcriptional co-activators, CBP and p300. Previous studies have used antibody blocking experiments to provide evidence that CBP and p300 play a role in mediating the ability of PRL promoter to respond to cAMP (134, 197). Our studies with a GAL4-p300 fusion provide evidence that the transcriptional activity of p300 can be stimulated by elevated cAMP in a MAPK-dependent manner. Thus, it is possible that CBP/p300 is constitutively present at the PRL promoter and that transcriptional activity of CBP/p300 is modulated by several signaling pathways including cAMP which converge on MAPK pathway. MAPK has been shown to directly phosphorylate CBP/p300 in vitro (204) and this in vitro phosphorylation has been demonstrated to enhance the in vitro histone acetyltransferase activity of CBP/p300 (205). It is possible that by phosphorylation of CBP/p300 , MAPK may activate the histone acetyltransferase activity of CBP/p300 in vivo, thereby, increasing the transcription of the prolactin gene. This could be tested in vivo by chromatin immunoprecipitation assays in which specific antibodies for acetylated histones are used to immunoprecipitate the prolactin promoter after treatment of cells with cAMP and PD98059. These experiments could determine if cAMP induces MAPK-dependent acetylation of histones on the prolactin promoter. Alternatively, Xu et al. (134) have suggested that activation of growth factor or cAMP pathways leads to recruitment of CBP/p300 to the PRL promoter. Additional studies assessing the recruitment of CBP/p300 to the PRL promoter in GH_3 cells are required to distinguish between these two models. The recent development of chromatin immunoprecipitation assays should provide an appropriate technology to address this question.

SUMMARY AND CONCLUSIONS

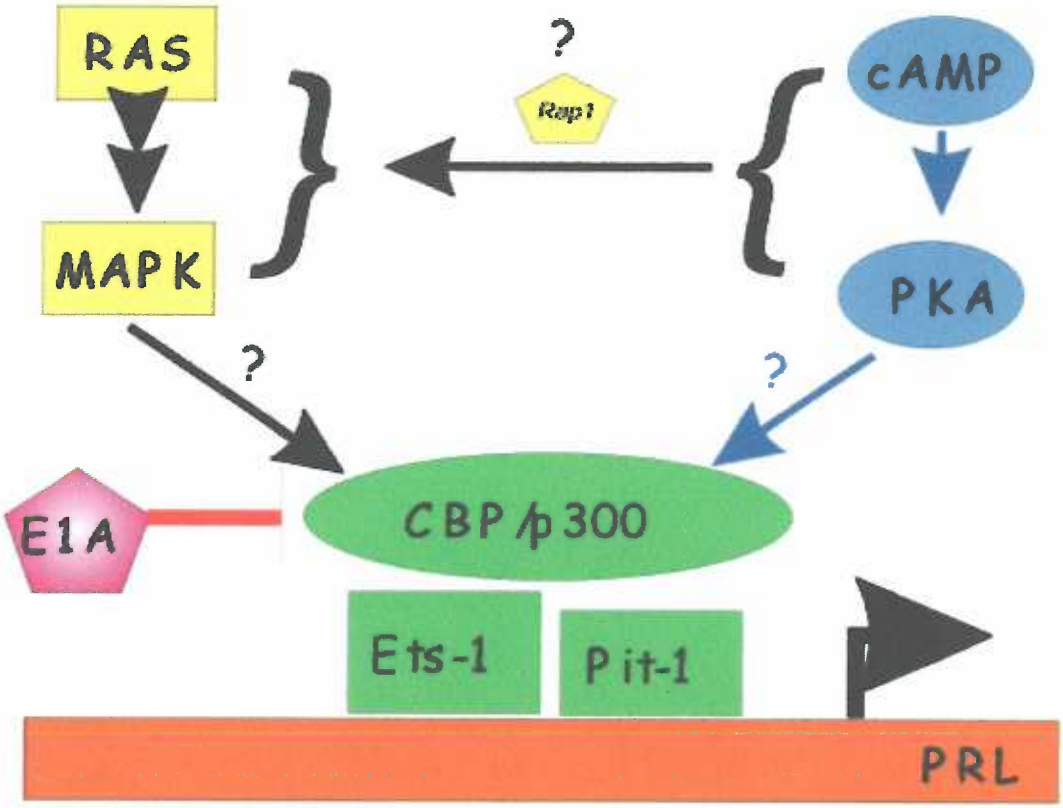
This thesis has explored the involvement of p300 in Pit-1/Ets-1 dependent prolactin promoter activity. It has also examined the effects of cAMP on mitogen activated protein kinase mediation of activation of the prolactin gene. In the first part of this thesis, we provided evidence for the involvement of CBP/p300 in activation of the prolactin promoter. A mammalian expression vector for p300 was found to enhance Pit-1/Ets-1 dependent activation of the proximal prolactin promoter. An in vitro binding assay demonstrated that p300 could bind to a proximal prolactin promoter DNA element in a Pit-1 and/or Ets-1 dependent manner. This suggested that the p300 induced enhancement of the reporter gene activation may be a result of direct recruitment of p300 to the prolactin promoter in the presence of Pit-1 and/or Ets-1. In order to further dissect the mechanism of p300 enhancement of the prolactin promoter and to determine if specific structural domains were responsible for its activity, we constructed several p300 deletion mutants and tested their activities in transient transfection assays. Unfortunately, none of the deletion mutants appeared to exhibit markedly different activities than wild type p300. Thus, in our hands, no one structural domain in particular was responsible for the activation of the prolactin promoter. However, the modest ability of p300 to enhance activation of the prolactin promoter substantially limited further interpretation of these studies. To determine if endogenous CBP/p300 was required for the mediation of Pit-1/Ets-1 dependent expression of the prolactin promoter we used an expression vector for the adenovirus 12S E1A protein. These results demonstrated that E1A greatly reduced the ability of Pit-1 and/or Ets-1 to activate expression of the prolactin promoter in COS-1 cells. E1A also completely blocked activation of the prolactin promoter by EGF/Ras while only partially blocking activation by forskolin/PKA in GH3 cells. These studies suggest the possibility of both a CBP/p300-independent

and a CBP/p300-dependent component of the forskolin/PKA pathway. As basal activation of the prolactin promoter was not affected by E1A, it seems likely that basal expression of prolactin in GH3 cells is not dependent on CBP/p300 activity.

In the second part of this thesis, we provided evidence that cAMP can activate the MAPK signaling cascade which contributes to the activation of the prolactin promoter in GH3 pituitary lactotroph cells. Elevation of cAMP levels by treatment of GH3 cells with forskolin led to the activation of MAPK. Inhibition of MAPK activation by the specific inhibitor, PD98059, reduced cAMP induced activation of the prolactin promoter. Thus MAPK appears to be required for full responsiveness of the prolactin promoter to cAMP. Mutation of Ets factor binding sites within the proximal prolactin promoter reduced responsiveness to TRH and EGF, but not to cAMP. Thus, although several signaling pathways appear to converge at MAPK, not all require Ets factors for activation of the prolactin promoter.

The data presented in this thesis may allow for the extrapolation of a possible model of prolactin promoter activation in response to different signaling pathways (Fig 1). This model shows activation of the prolactin promoter by two different signaling pathways which converge on CBP/p300. The Ras/MAPK pathway activates CBP/p300 in an E1a sensitive manner. The cAMP/PKA pathway activates CBP/p300 through both an E1a sensitive and insensitive pathway. The E1A sensitive component of the cAMP/PKA pathway may be a result of cross-talk with the Ras/MAPK pathway, mediated in part by Rap1 activation (as determined by Paul Kievit in the Maurer lab. Unpublished observations). The CBP/p300 activity blocked by E1A may actually be a CBP/p300 associated factor other than CBP/p300 itself, perhaps P/CAF. Future experiments investigating the role of P/CAF in the regulated expression of the prolactin promoter should shed light on this highly speculative model.

Fig.1. A Possible Model for CBP/p300 Dependent Expression of the Prolactin Promoter in Response to Different Signaling Pathways. CBP/p300 is recruited to the prolactin promoter in a Pit-1 and/or Ets-1 dependent manner and is activated by both Ras and cAMP. The Ras pathway is sensitive to inhibition by E1A. The cAMP pathway is composed of two separate pathways, one sensitive and the other insensitive to inhibition by E1A. The E1A sensitive component of the cAMP pathway is accomplished via cross-talk with the Ras pathway, possibly mediated through Rap1.


RAS
?
Rap1
cAMP
MAPK
PKA
?
?
E1A
CBP/p300
Ets-1
Pit-1
PRL

REFERENCES

1. Sticker P, G.R. (1928) Action du lobe anterieur de l'hypophyse sur la montee' laiteuse. C R Soc Biol 99, 1978-1980
2. Riddle O, B.R., Dykshorn SW (1933) The preparation, identification and assay of prolactin- a hormone of the anterior pituitary. Am J Physiol 105, 191-216
3. Astwood, E. (1941) The regulation of corpus luetum function by hypophysial luteotropin. Endocrinology 29, 309-319
4. Li CH, D.J., Lo TB, Pankov YM, Schmidt KD (1969) Amino acid sequence of ovine lactogenic hormone. Nature 224, 695-696
5. Hwang P, G.H., Friesen H (1971) A radioimmunoassay for human prolactin. Proc Natl Acad Sci USA 68,
6. Lewis UJ, S.R., Seavey BK (1971) Human prolactin: isolation and some properties. Biochem Biophys Res Commun 44, 1169-1176
7. Gubbins, E.J., R.A. Maurer, M. Lagrimini, C.R. Erwin and J.E. Donelson (1980) Structure of the rat prolactin gene. J. Biol. Chem. 255, 8655-8662
8. Miller WL, C.D., Baxter JD, Martial JA (1981) Cloning of bovine prolactin cDNA and evolutionary implications of its sequence. DNA 1, 37-50
9. Cooke NE, C.D., Shine J, Baxter JD, Martial JA (1981) Human prolactin. cDNA structural analysis and evolutionary comparisons. J. Biol. Chem. 256, 4007-4016
10. Richardson, B.P. (1973) Evidence for a physiological role of prolactin in osmoregulation in the rat after its inhibition by 2-bromo-ergokryptine. Br. J. Pharmacol. 47, 623P-624P
11. Mainoya, J.R., H.A. Bern and J.W. Regan (1974) Influence of ovine prolactin on transport of fluid and sodium chloride by the mammalian intestine and gall bladder. J Endocrinol 63, 311-317

12. Josimovich, J.B., K. Merisko and L. Boccella (1977) Amniotic prolactin control over amniotic and fetal extracellular fluid water and electrolytes in the rhesus monkey. Endocrinology 100, 564-570

13. Perez-Villamil, B., E. Bordiu and M. Puente-Cueva (1992) Involvement of physiological prolactin levels in growth and prolactin receptor content of prostate glands and testes in developing male rats. J Endocrinol 132, 449-459

14. Rynikova, A., J. Koppel, S. Kuchar, S. Cikos and S. Mozes (1988) Effects of ovine prolactin in infant rats. Exp Clin Endocrinol 92, 241-244

15. Hamosh, M. and P. Hamosh (1977) The effect of prolactin on the lecithin content of fetal rabbit lung. J Clin Invest 59, 1002-1005

16. Machida, T., M. Taga and H. Minaguchi (1990) Effect of prolactin (PRL) on lipoprotein lipase (LPL) activity in the rat fetal liver. Asia Oceania J Obstet Gynaecol 16, 261-265

17. Bridges, R.S., R. DiBiase, D.D. Loundes and P.C. Doherty (1985) Prolactin stimulation of maternal behavior in female rats. Science 227, 782-784

18. Bridges, R.S., M. Numan, P.M. ronsheim, P.E. Mann and C.E. Lupini (1990) Central prolactin infusions stimulate maternal behavior in steroid-treated, nulliparous female rats. Proc. Natl. Acad. Sci. U.S.A. 87, 8003-8007

19. Bridges, R.S., M.C. Robertson, R.P. Shiu, J.D. Sturgis, B.M. Henriquez and P.E. Mann (1997) Central lactogenic regulation of maternal behavior in rats: steroid dependence, hormone specificity, and behavioral potencies of rat prolactin and rat placental lactogen I. Endocrinology 138, 756-763

20. Guyette, W.A., R.J. Matusik and J.M. Rosen (1979) Prolactin-mediated transcriptional and post-transcriptional control of casein gene expression. Cell 17, 1013-1023

21. Oppat, C.A. and J.A. Rillema (1988) Characteristics of the early effect of prolactin on lactose biosynthesis in mouse mammary gland explants. Proc Soc Exp Biol Med 188, 342-345

22. Waters, S.B. and J.A. Rillema (1988) Effect of prolactin on enzymes of lipid biosynthesis in mammary gland explants. Am J Physiol 255, E567-571

23. Berczi, I., E. Nagy, K. Kovacs and E. Horvath (1981) Regulation of humoral immunity in rats by pituitary hormones. Acta Endocrinol (Copenh) 98, 506-513

24. McMurray, R., D. Keisler, K. Kanuckel, S. Izui and S.E. Walker (1991) Prolactin influences autoimmune disease activity in the female B/W mouse. J Immunol 147, 3780-3787

25. Lahat, N., A. Miller, R. Shtiller and E. Touby (1993) Differential effects of prolactin upon activation and differentiation of human B lymphocytes. J Neuroimmunol 47, 35-40

26. Bernton, E.W., M.S. Meltzer and J.W. Holaday (1988) Suppression of macrophage activation and T-lymphocyte function in hypoprolactinemic mice. Science 239, 401-404

27. Edwards, C.K.d., S.M. Ghiasuddin, J.M. Schepper, L.M. Yunger and K.W. Kelley (1988) A newly defined property of somatotropin: priming of macrophages for production of superoxide anion. Science 239, 769-771

28. Fu, Y.K., S. Arkins, G. Fuh, B.C. Cunningham, J.A. Wells, S. Fong, M.J. Cronin, R. Dantzer and K.W. Kelley (1992) Growth hormone augments superoxide anion secretion of human neutrophils by binding to the prolactin receptor. J Clin Invest 89, 451-457

29. Blackwell, R.E. (1992) Hyperprolactinemia. Evaluation and management. Endocrinol Metab Clin North Am 21, 105-124

30. Sobrinho, L.G. (1993) The psychogenic effects of prolactin. Acta Endocrinol 129,

31. Bhatavdeker J, P.D., Ghosh N, Vora H, Shah N, Karelia N, Balar D, Chikhlikar P, Dave R. (1994) Inerrelation of prolactin and its receptor in carcinoma of colon and rectum: a preliminary report. J Surg Oncol 55, 246-249

32. Bhatavdeker J, P.D., Shah N, Karelia N, Vora H, Ghosh N, Suthar T, Balar D. (1995) Prognostic value of insulin-like growth factor-1 in patients with colon/rectal cancer: correlation with plasma prolactin. Eur J Surg Oncol 21, 23-26

33. Kiss R, d.L.Y., L'Hermite-Baleriaux M, L'Hermite M, Paridaens R, Danguy A, Pasteels J. (1987) Effect of prolactin and estradiol on cell proliferation in the uterus and the MXT mouse mammary neoplasm. J Natl Cancer Inst 78, 993-998

34. Walker A, M.D., Saraiya S, Ho T, Garewel H, Wilson J. (1995) Prolactin-immunoglobulin G complexes from human serum act as costimulatory ligands causing proliferation of malignant B lymphocytes. Proc Natl Acad Sci USA 92,

35. Gout P, B.C., Noble R. (1980) Prolactin stimulated growth of cell cultures established from malignant Nb rat lymphocytes. Cancer Res 40, 2433-2436

36. McMurray R, K.D., Kanuckel K, Izui S, Walker S (1991) Prolactin influences autoimmune disease activity in the female B/W mouse. J immunol 147, 3780-3787

37. El-Garf A, S.S., Shaaraway M, Zaki S, Anwer S (1996) Prolactin hormone in juvenile systemic lupus erythematosus: a possible relationship to disease avtivity and CNS manifestations. J Rheumatol 23, 374-377

38. Riskind P, M.L., Doolittle T, Hauser S. (1991) The role of prolactin in autoimmune demyelination: suppression of experimental allergic encephalomyelitis by bromocritptine. Ann Neurol 29, 542-547

39. McMurray R, A.S., Pepmueller P, Keisler D, Cassidy J. (1995) Elevated serum prolactin levels in children with juvenile rheumatiod arthritis and antinuclear antibody seropositivity. J Reumatol 22, 1577-1580

40. Neidhart M, F.E. (1992) Hyperprolactinaemia in hypophysectomized or intact male rats and the development of adjuvant arthritis. Immunology 77, 449-455

41. Hinterberger-Fisher M, K.P., Spona J, Sebesta C, Tiefengraber E, Habertheuer K, Ruckser R, Schmid A, Kalhs P,Lechner K, Hinterberger W. (1994) Prolactin: a possible mediator of graft vs. host disease following allogenic bone marrow transplantation in humans. Bone marrow transplant 14, 403-406

42. Wu, H., R. Devi and W.B. Malarkey (1996) Expression and localization of prolactin messenger ribonucleic acid in the human immune system. Endocrinology 137, 349-353

43. Montgomery, D.W., G.K. Shen, E.D. Ulrich, L.L. Steiner, P.R. Parrish and C.F. Zukoski (1992) Human thymocytes express a prolactin-like messenger ribonucleic acid and synthesize bioactive prolactin-like proteins. Endocrinology 131, 3019-3026

44. O'Neal, K.D., D.W. Montgomery, T.M. Truong and L.Y. Yu-Lee (1992) Prolactin gene expression in human thymocytes. Mol Cell Endocrinol 87, R19-R23

45. Delhase, M., P. Vergani, A. Malur, B. Velkeniers, E. Teugels, J. Trouillas and E.L. Hooghe-Peters (1993) Pit-1/GHF-1 expression in pituitary adenomas: further analogy between human adenomas and rat SMtTW tumours. J Mol Endocrinol 11, 129-139

46. Wu, H., R. Devi and W.B. Malarkey (1996) Expression and localization of prolactin messenger ribonucleic acid in the human immune system. Endocrinology 137, 349-353

47. Riddick, D.H., A.A. Luciano, W.F. Kusmik and I.A. Maslar (1978) De novo synthesis of prolactin by human decidua. Life Sci 23, 1913-1921

48. Clements, J., P. Whitfeld, N. Cooke, D. Healy, B. Matheson, J. Shine and J. Funder (1983) Expression of the prolactin gene in human decidua-chorion. Endocrinology 112, 1133-1134

49. Chapitis, J., D.H. Riddick, L.M. Betz, J.R. Brumsted, M. Gibson, J.C. Prior and P.W. Gout (1988) Physicochemical characterization and functional activity of fibroid prolactin produced in cell culture. Am. J. Obstet. Gynecol. 158, 846-853

50. Gellersen, B., A. Bonhoff, N. Hunt and H.G. Bohnet (1991) Decidual-type prolactin expression by the human myometrium. Endocrinology 129, 158-168

51. Nolin, J.M. and R.J. Witorsch (1976) Detection of endogenous immunoreactive prolactin in rat mammary epithelial cells during lactation. Endocrinology 99, 949-958

52. Malven, P.V. and T.W. Keenan (1983) Immunoreactive prolactin in subcellular fractions from bovine mammary tissue. J Dairy Sci 66, 1237-1242

53. Richards, R.G. and S.M. Hartman (1996) Human dermal fibroblast cells express prolactin in vitro. J Invest Dermatol 106, 1250-1255

54. Laverriere, J.N., A. Morin, A. Tixier-Vidal, A.T. Truong, D. Gourdji and J.A. Martial (1983) Inverse control of prolactin and growth hormone gene expression: effect of thyroliberin on transcription and RNA stabilization. EMBO J. 2, 1493-1500

55. Murdoch, G.H., R. Franco, R.M. Evans and M.G. Rosenfeld (1983) Polypeptide hormone regulation of gene expression. thyrotropin-releasing hormone rapidly stimulates both transcription of the prolactin gene and the phosphorylation of a specific nuclear protein. J. Biol. Chem. 258, 15329-15335

56. Maurer, R.A. (1982) Estradiol regulates the transcription of the prolactin gene. J. Biol. Chem. 257, 2133-2136

57. Rosenfeld, M.G., C. Nelson, E.B. Crenshaw, III, H.P. Elsholtz, S.A. Lira, H.J. Mangalam, R. Franco, M. Waterman, C. Weinberger, S.M. Hollenberg, V. Giguere, E. Ong and R.M. Evans (1987) Developmental and hormonal regulation of neuroendocrine gene transcription. Recent Progress in Horm. Res. 43, 499-534

58. Stanley, F. (1988) Stimulation of prolactin gene expression by insulin. J. Biol. Chem. 263, 13444-13448

59. Maurer, R.A. (1981) Transcriptional regulation of the prolactin gene by ergocryptine and cyclic AMP. Nature 294, 94-97

60. Elsholtz, H.P., A.M. Lew, P.R. Albert and V.C. Sundmark (1991) Inhibitory control of prolactin and Pit-1 gene promoters by dopamine. dual signaling pathways required for D2 receptor-regulated expression of the prolactin gene. J. Biol. Chem. 266, 22919-22925

61. McChesney, R., S.C. Sealfon, M. Tsutsumi, K.W. Dong, J.L. Roberts and C. Bancroft (1991) Either isoform of the dopamine D2 receptor can mediate dopaminergic expression of the rat prolactin promoter. Mol. Cell. Endocrinol. 79, R1-R7

62. Keech, C.A. and A. Gutierrez-Hartmann (1989) Analysis of rat prolactin promoter sequences that mediate pituitary-specific and 3',5'-cyclic adenosine monophosphate-regulated gene expression in vivo. Mol. Endocrinol. 3, 832-839

63. Iverson, R.A., K.H. Day, M. d'Emden, R.N. Day and R.A. Maurer (1990) Clustered point mutation analysis of the rat prolactin promoter. Mol. Endocrinol. 4, 1564-1571

64. Rajnarayan, S., M. Chiono, L.M. Alexander and A. Gutierrez-Hartmann (1995) Reconstitution of protein kinase A regulation of the rat prolactin promoter in HeLa nonpituitary cells: identificaiton of both GHF-1/Pit-1-dependent and -independent mechanisms. Mol. Endocrinol. 9, 502-512

65. Montminy, M.R. and L.M. Bilezikjian (1987) Binding of a nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature 328, 175-178

66. Liang, J., K.E. Kim, W.E. Schoderbek and R.A. Maurer (1992) Characterization of a nontissue-specific 3',5'-cyclic adenosine monophosphate-responsive element in the proximal region of the rat prolactin gene. Mol. Endocrinol. 6, 885-892

67. Nelson, C., E.B. Crenshaw, III, R. Franco, S.A. Lira, V.R. Albert, R.M. Evans and M.G. Rosenfeld (1986) Discrete cis-active genomic sequences dictate the pituitary cell type-specific expression of rat prolactin and growth hormone genes. Nature 322, 557-562

68. Gutierrez-Hartmann, A., S. Siddiquid and S. Loukin (1987) Selective transcription and DNase I protection of the rat prolactin gene by GH_3 pituitary cell-free extracts. Proc. Natl. Acad. Sci. U.S.A. 84, 5211-5215

69. Lufkin, T. and C. Bancroft (1987) Identification by cell fusion of gene sequences that interact with positive trans-acting factors. Science 237, 283-286

70. Cao, Z., E.A. Barron, A.J. Carrillo and Z.D. Sharp (1987) Reconstitution of cell-type-specific transcription of the rat prolactin gene in vitro. Mol. Cell. Biol. 7, 3402-3408

71. Schuster, W.A., M.N. Treacy and F. Martin (1988) Tissue specific trans-acting factor interaction with proximal rat prolactin gene promoter sequences. EMBO J. 7, 1721-1733

72. Day, R.N. and R.A. Maurer (1989) The distal enhancer region of the rat prolactin gene contains elements conferring response to multiple hormones. Mol. Endocrinol. 3, 3-9

73. Nelson, C., V.R. Albert, H.P. Elsholtz, L.I.-W. Lu and M.G. Rosenfeld (1988) Activation of cell-specific expression of rat growth hormone and prolactin genes by a common transcription factor. Science 239, 1400-1405

74. Lufkin, T., A.E. Jackson, W.T. Pan and C. Bancroft (1989) Proximal rat prolactin promoter sequences direct optimal, pituitary cell-specific transcription. Mol. Endocrinol. 3, 559-566

75. Mangalam, H.J., V.R. Albert, H.A. Ingraham, M. Kapiloff, L. Wilson, C. Nelson, H. Elsholtz and M.G. Rosenfeld (1989) A pituitary POU domain protein, Pit-1, activates

both growth hormone and prolactin promoters transcriptionally. Genes Dev. 3, 946-958

76. Crenshaw, E.B., III, K. Kalla, D.M. Simmons, L.W. Swanson and M.G. Rosenfeld (1989) Cell-specific expression of the prolactin gene in transgenic mice is controlled by synergistic interactions between promoter and enhancer elements. Genes Dev. 3, 959-972

77. Ingraham, H.A., R. Chen, H.J. Mangalam, H.P. Elsholtz, S.E. Flynn, C.R. Lin, D.M. Simmons, L. Swanson and M.G. Rosenfeld (1988) A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. Cell 55, 519-529

78. Bodner, M., J.-L. Castrillo, L.E. Theill, T. Deerinck, M. Ellisman and M. Karin (1988) The pituitary-specific transcription factor GHF-1 is a homeobox-containing protein. Cell 55, 505-518

79. Herr, W., R.A. Sturm, R.G. Clerc, L.M. Corcoran, D. Baltimore, P.A. Sharp, H.A. Ingraham, M.G. Rosenfeld, M. Finney, G. Ruvkun and H.R. Horvitz (1988) The POU domain: a large conserved region in the mammalian pit-1, oct-1, oct-2, and Caenorhabditis elegans unc-86 gene products. Genes Dev. 2, 1513-1516

80. Theill, L.E., J.-L. Castrillo, D. Wu and M. Karin (1989) Dissection of functional domains of the pituitary-specific transcription factor GHF-1. Nature 342, 945-948

81. Ingraham, H.A., S.E. Flynn, J.W. Voss, V.R. Albert, M.S. Kapiloff, L. Wilson and M.G. Rosenfeld (1990) The POU-specific domain of Pit-1 is essential for sequence-specific, high affinity DNA binding and DNA-dependent Pit-1-Pit-1 interactions. Cell 61, 1021-1033

82. Sturm, R.A. and W. Herr (1988) The POU domain is a bipartite DNA-binding structure. Nature 336, 601-604

83. Jacobson, E.M., P. Li, A. Leon-del-Rio, M.G. Rosenfeld and A.K. Aggarwal (1997) Structure of Pit-1 POU domain bound to DNA as a dimer: unexpected arrangement and flexibility. Genes Dev. 11, 198-212

84. Klemm, J.D., M.A. Ropuld, R. Aurora, W. Herr and C.O. Pabo (1994) Crystal structure of the Oct-1 POU domain bound to an octamer site: DNA recognition with tethered DNA-binding modules. Cell 77, 21-32

85. Simmons, D.M., J.W. Voss, H.A. Ingraham, J.M. Holloway, R.S. Broide, M.G. Rosenfeld and L.W. Swanson (1990) Pituitary cell phenotypes involve cell-specific Pit-1 mRNA translation and synergistic interactions with other classes of transcription factors. Genes Dev. 4, 695-711

86. Steinfelder, H.J., P. Hauser, Y. Nakayama, S. Radovick, J.H. McClaskey, T. Taylor, B.D. Weintraub and F.E. Wondisford (1991) Thyrotropin-releasing hormone regulation of human TSHβ expression: role of a pituitary specific transcription factor (Pit-1/GHF-1) and potential interaction with a thyroid hormone inhibitory element. Proc. Natl. Acad. Sci. U.S.A. 88, 3130-3134

87. Steinfelder, H.J., S. Radovick, M.A. Mroczynski, P. Hauser, J.H. McClaskey, B.D. Weintraub and F.E. Wondisford (1992) Role of a pituitary-specific transcription factor (Pit-1/GHF-1 or a closely related protein in cAMP regulation of human thyrotropin-β gene expression. J. Clin. Invest. 89, 409-419

88. Li, S., E.B. Crenshaw, III, E.J. Rawson, D.M. Simmons, L.W. Swanson and M.G. Rosenfeld (1990) Dwarf locus mutants lacking three pituitary cell types result from mutations in the POU-domain gene *pit-1*. Nature 347, 528-533

89. Andersen B, P.R., Jenne K, Sorenson M, Lin SC , Bartke A, Rosenfeld MG (1995) The AMes dwarf gene is required for Pit-1 gene activation. Dev Biol 172, 495-503

90. Cohen LE, W.F., Radovick S (1996) Role of Pit-1 in the gene expression of growth hormone, prolactin, and thyrotropin. Endocrinol Metab Clin North AM sept;25, 523-540

91. Cohen, L.E., F.E. Wondisford, A. Salvatoni, M. Magnie, F. Brucker-Davis, B.D. Weintraub and S. Radovick (1995) A "hot spot" in the Pit-1 gene responsible for combined pituitary hormone deficiency: clinical and moelcular correlates. Endocrinology 80, 679-684

92. Radovick, S., M. Nations, Y. Du, L.A. Berg, B.D. Weintraub and F.E. Wondisford (1992) A mutation in the POU-homeodomain of Pit-1 responsible for combined pituitary hormone deficiency. Science 257, 1115-1118

93. Konzak, K.E. and D.D. Moore (1992) Functional isoforms of Pit-1 generated by alternative messenger RNA splicing. Mol. Endocrinol. 6, 241-247

94. Haugen, B.R., W.M. Wood, D.F. Gordon and E.C. Ridgway (1993) A thyrotrope-specific variant of Pit-1 transactivates the thyrotropin β promoter. J. Biol. Chem. 268, 20818-20824

95. Morris, A.E., R.E. McChesney, C. Bancroft and L.A. Chasin (1992) An alternatively spliced Pit-1 isoform altered in its ability to trans-activate. Nuc. Acids Res. 20, 1355-1361

96. Diamond, S.E. and A. Gutierrez-Hartmann (1996) A 26-amino acid insertion domain defines a functional transcription switch motif in Pit-1β. J. Biol. Chem. 271, 28925-28932

97. Haugen, B.R., D.F. Gordon, A.R. Nelson, W.M. Wood and E.C. Ridgway (1994) The combination of Pit-1 and Pit-1T have a synergistic stimulatory effect on the thyrotropin β-subunit promoter but not the growth hormone or prolactin promoters. Mol. Endocrinol. 8, 1574-1582

98. Watson, D.K., M.J. McWilliams, P. Lapis, J.A. Lautenberger, C.W. Schweinfest and T.S. Papas (1988) Mammalian *ets-1* and *ets-2* genes encode highly conserved proteins. Proc. Natl. Acad. Sci. U.S.A. 85, 7862-7866

99. Rao, V.N. and E.S. Reddy (1992) A divergent *ets*-related protein, Elk-1, recognizes similar c-*ets*-1 proto-oncogene target sequences and acts as a transcriptional activator. Oncogene 7, 65-70

100. Dalton, S. and R. Treisman (1992) Characterization of SAP-1, a protein recruited by serum response factor to the c-fos serum response element. Cell 68, 597-612

101. Thompson, C.C., T.A. Brown and S.L. McKnight (1991) Convergence of Ets- and notch-related structural motifs in a heteromeric DNA binding complex. Science 253, 762-768

102. frampton J, L.A., Gibson T, Graf T (1989) DNA-binding domain ancestry. Nature 9, 134

103. Anton IA, F.J. (1988) Tryptophans in myb proteins. Nature 29, 719

104. Donaldson L, p.J., Graves B, McIntosh L (1996) Solution structure of the ETS domain from murine ETS-1: a winged helix-turn-helix DNA binding motif. EMBO J 15, 124-134

105. Janknecht, R. and A. Nordheim (1992) Elk-1 protein domains required for direct and SRF-assisted DNA-binding. Nucleic Acids Res. 20, 3317-3324

106. Woods, D.B., J. Ghysdael and M.J. Owen (1992) Identification of nucleotide preferences in DNA sequences recognised specifically by c-Ets-1 protein. Nucleic Acids Res. 20, 699-704

107. Wasylyk, B., C. Wasylyk, P. Flores, A. Begue, D. Leprince and D. Stehelin (1990) The c-ets proto-oncogenes encode transcription factors that cooperate with c-fos and c-jun for transcriptional activation. Nature 346, 191-193

108. Yang, B.S., C.A. Hauser, G. Henkel, M.S. Colmlan, C. Van Beveren, K.J. Stacey, D.A. Hume, R.A. Maki and M.C. Ostrowski (1996) Ras-mediated phosphorylation of a conserved threonine residue enhances the transactivation activities of c-Ets1 and c-Ets2. Mol. Cell. Biol. 16, 538-547

109. Gegonne, A., R. Rosselut, R.-A. Bailly and J. Ghysdael (1993) Synergistic activation of the HTLV1 LTR Ets-responsive region by transcription factors Ets-1 and Sp-1. EMBO J. 12, 1169-1178

110. Conrad, K.E., J.M. Oberwetter, R. Vaillancourt, G.L. Johnson and A. Gutierrez-Hartmann (1994) Identification of the functional components of the Ras signaling pathway regulating pituitary cell-specific gene expression. Mol. Cell. Biol. 14, 1553-1565

111. Wang, Y.-H. and R.A. Maurer (1999) A role for the mitogen-activated protein kinase in mediating the ability of thyrotropin-releasing hormone to stimulat the prolactin promoter. Mol. Endocrinol. 13, 1094-1104

112. MacLeod, K., D. Leprince and D. Stehelin (1992) The *ets* gene family. Trends Biochem. Sci. 17, 251-256

113. Bradford, A.P., K.E. Conrad, C. Wasylyk, B. Wasylyk and A. Gutierrez-Hartmann (1995) Functional interaction of c-Ets-1 and GHF-1/Pit-1 mediates Ras activation of pituitary-specific gene expression: mapping of the essential c-Ets-1 domain. Mol. Cell. Biol.I 15, 2849-2857

114. Yee S, B.P. (1985) Detection of cellular proteins associated with human adenovirus type 5 region 1A polypeptides. virology 147, 142-153

115. Harlow E, W.B., Franza J, Schley C (1986) association of adenovirus early region 1A with cellular polypeptides. Mol Cell Biol 6, 1579-1589

116. Bannister, A.J. and T. Kouzarides (1995) CBP-induced stimulation of c-Fos activity is abrogated by E1A. EMBO J. 14, 4758-4762

117. Egan, C., T.N. Jelsma, J.A. Howe, S.T. Bayley, B. Ferguson and P.E. Branton (1988) Mapping of cellular protein-binding sites on the products of early-region 1A of human adenovirus type 5. Mol Cell Biol 8, 3955-3959

118. Montminy, M.R., K.A. Sevarino, J.A. Wagner, G. Mandel and R.H. Goodman (1986) Identification of a cyclic-AMP-responsive element within the rat somatostatin gene. Proc. Natl. Acad. Sci. U.S.A. 83, 6682-6686

119. Comb, M., N.C. Birnberg, A. Seasholtz, E. Herbert and H.M. Goodman (1986) A cyclic AMP- and phorbol ester-inducible DNA element. Nature 323, 353-356

120. Gonzalez, G.A., K.K. Yamamoto, W.H. Fischer, D. Karr, P. Menzel, W. Biggs, III, W.W. Vale and M.R. Montminy (1989) A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. Nature 337, 749-752

121. Hoeffler, J.P., T.E. Meyer, Y. Yun, J.L. Jameson and J.F. Habener (1988) Cyclic AMP-responsive DNA-binding protein: structure based on a cloned placental cDNA. Science 242, 1430-1433

122. Gonzalez, G.A. and M.R. Montminy (1989) Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59, 675-680

123. Chrivia, J.C., R.P.S. Kwok, N. Lamb, M. Hagiwara, M.R. Montminy and R.H. Goodman (1993) Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature 365, 855-859

124. Arany, Z., W.R. Sellers, D.M. Livingston and R. Eckner (1994) E1A-associated p300 and CREB-associated CBP belong to a conserved family of coactivators. Cell 77, 799-800

125. Haynes, S.R., C. Dollard, F. Winston, S. Beck, J. Trowsdale and I.B. Dawid (1992) The bromodomain: a conserved sequence found in human, *Drosophila* and yeast proteins. Nuc. Acids Res. 20, 2603

126. Tamkun J, D.R., Scott M, Kissinger M, Pattatucci A, Kaufman T, Kennison J. (1992) Brahma: a regulator of drosophila homeotic genes structurally related to the yeast transcriptional activator SNF2/SWI2. Cell 68, 561-572

127. Syntichaki P, T.I., Thireos G (2000) The Gcn5 Bromodomain co-ordinates nucleosome remodelling. Nature 404, 414-417

128. Bannister, A.J. and T. Kouzarides (1996) The CBP co-activator is a histone acetyltransferase. Nature 384, 641-643

129. Ogryzko, V.V., R.L. Schlitz, V. Russanova, B.H. Howard and Y. Nakatani (1996) The transcriptional coactivators p300 and CBP are histone acetyltransferances. Cell 87, 953-959

130. Allfrey, V.G. (1966) Structural modifications of histones and their possible role in the regulation of ribonucleic acid synthesis. Proc Can Cancer Conf 6, 313-335

131. Lee, D.Y., J.J. Hayes, D. Pruss and A.P. Wolffe (1993) A positive role for histone acetylation in transcription factor access to nucleosomal DNA. Cell 72, 73-84

132. Howard, P.W. and R.A. Maurer (1995) A composite Ets/Pit-1 binding site in the prolactin gene can mediate transcriptional responses to multiple signal transduction pathways. J. Biol. Chem. 270, 20930-20936

133. Bradford, A.P., C. Wasylyk, B. Wasylyk and A. Gutierrez-Hartmann (1997) Interaction of Ets-1 and the POU-homeodomain protei GHF-1/Pit-1 reconstitutes pituitary-specific gene expression. Mol. Cell. Biol. 17, 1065-1074

134. Xu, L., R.M. Lavinsky, J.S. Dasen, S.E. Flynn, E.M. McInerney, T.-M. Mullen, T. Heizel, D. Szeto, E. Korzus, R. Kurokawa, A.K. Aggarwal, D.W. Rose, C.K. Glass and

M.G. Rosenfeld (1998) Signal-specific co-activator domain requirements for Pit-1 activation. Nature 395, 301-306

135. Tolon, R.M., A.I. Castilio and A. Aranda (1998) Activation of the prolactin gene by peroxisome proliferator-activated receptor-α appears to be DNA binding-independent. J. Biol. Chem. 273, 26652-26661

136. Yang, C., L.H. Shapiro, M. Rivera, A. Kumar and P.K. Brindle (1998) A role for CREB binding protein and p300 transcriptional coactivators in Ets-1 transactivation functions. Mol. Cell. Biol. 18, 2218-2229

137. Shapiro, L.H. (1995) Myb and Ets proteins cooperate to transactivate an early myloid gene. J. Biol. Chem. 270, 8763-8771

138. Dai, P., H. Akimaru, Y. Tanaka, D.-X. Hou, T. Yasukawa, C. Kanei-Ishii, T. Takahashi and S. Ishii (1996) CBP as a transcriptional coactivator of c-Myb. Genes Dev. 10, 528-540

139. Jayaraman, G., R. Srinivas, C. Duggan, E. Ferreira, S. Swaminathan, K. Somasundaram, J. Williams, C. Hauser, M. Kurkinen, R. Dhar, S. Weitzman, G. Guttice and B. Thimmapaya (1999) p300/cAMP-responsive element-binding protein interactions with Ets-1 and Ets-2 in the transcriptional activation of the human stromelysin promoter. J. Biol. Chem. 274, 17342-17352

140. Buttice, G. and M. Kurkinen (1993) A polyomavirus enhancer A-binding protein-3 site and Ets-2 protein have a major role in the 12-O-tetradecanoylphorbol-13-acetate response of the human stromelysin gene. J. Biol. Chem. 268, 7196-7204

141. Buttice G, Q.S., Kurkinen M (1991) The AP-1 site is required for the basal expression but is not necessary for TPA-response of the human stromelysin gene. Nucleic Acids Res 19, 3723-3731

142. Wasylyk C, G.A., Nicholson R, Wasylyk B (1991) The c-Ets oncoprotein activates the stromelysin promoter through the same elements as several non-nuclear oncoproteins. Embo J 5, 1127-1134

143. Bradford, A.P., K.E. Conrad, Tran, P.H., M.C. Ostrowski and A. Gutierrez-Hartmann (1996) GHF-1/Pit-1 functions as a cell-specific integrator of Ras signalling by targeting the Ras pathway to a composite Ets-1/GHF-1 response elements. J. Biol. Chem. 271, 24639-24648

144. Jayaraman, G., R. Srinivas, C. Duggan, E. Ferreira, S. Swaminathan, K. Somasundaram, J. Williams, C. Hauser, M. Kurkinen, R. Dhar, S. Weitzman, G. Buttice and B. Thimmapaya (1999) p300/cAMP-responsive element-binding protein interactions with Ets-1 and Ets-2 in the transcriptional activation of the human strmelysin promoter. J. Biol. Chem. 274, 17342-17352

145. Kraus, W.L., E.T. Manning and J.T. Kadonaga (1999) Biochemical analysis of distinct activation functions in p300 that enhance transcription initiation with chromatin templates. Mol Cell Biol 19, 8123-35

146. Lundblad, J.R., R.P.S. Kwok, M.E. Laurance, M.L. Harter and R.H. Goodman (1995) Adenoviral E1A-associated protein p300 as a functional homologue of the transcriptional co-activator CBP. Nature 374, 85-88

147. Arany, Z., D. Newsome, E. Oldread, D.M. Livingston and R. Eckner (1995) A family of transcriptional adaptor proteins targeted by the E1A oncoprotein. Nature 374, 81-84

148. Whyte, P., K.J. Buchkovich, J.M. Horowitz, S.H. Friend, M. Raybuck, R.A. Weinberg and E. Harlow (1988) Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product. Nature 334, 124-129

149. Mayol, X., X. Grana, A. Baldi, N. Sang, Q. Hu and A. Giordano (1993) Cloning of a new member of the retinoblastoma gene family (pRb2) which binds to the E1A transforming domain. Oncogene 8, 2561-2566

150. Wang, H.-G.H., E. Moran and P. Yaciuk (1995) E1A promotes assocation between p300 and pRB in multimeric complexes required for normal biological activity. J. Virol. 69, 7917-7924

151. Maurer, R.A. (1982) Adenosine 3',5'-monophosphate derivatives increase prolactin synthesis and prolactin messenger ribonucleic acid levels in ergrocryptine-treated pituitary cells. Endocrinology 110, 1957-1963

152. Day, R.N., J. Walder and R.A. Maurer (1989) A protein kinase inhibitor gene reduces both basal and multihormone-stimulated prolactin gene transcription. J. Biol. Chem. 264, 431-436

153. Maurer, R.A. (1989) Both isoforms of the cAMP-dependent protein kinase catalytic subunit can activate transcription of the prolactin gene. J. Biol. Chem. 264, 6870-6873

154. Ohmichi, M., T. Sawada, Y. Kanda, K. Koike, K. Hirota, A. Miyake and A.R. Saltiel (1994) Thyrotropin-releasing hormone stimulates MAP kinase activity in GH3 cells by divergent pathways. Evidence of a role for early tyrosine phosphorylation. J. Biol. Chem. 269, 3783-3788

155. Oberwetter, J.M., K.E. Conrad and A. Gutierrez-Hartmann (1993) The Ras and protein kinase C signaling pathways are functionally antagonistic in GH4 neuroendocrine cells. Mol Endocrinol 7, 915-923

156. Conrad, K.E. and A. Gutierrez-Hartmann (1992) The *ras* and protein kinase A pathways are mutually anatagonistic in regulating rat prolactin promoter activity. Oncogene 7, 1279-1286

157. Kamei, Y., L. Xu, T. Heinzel, J. Torchia, R. Kurokawa, B. Gloss, S.-C. Lin, R.A. Heyman, D.W. Rose, C.K. Glass and M.G. Rosenfeld (1996) A CBP integrator complex mediates transcriptional activation and AP-1 inhibition by nuclear receptors. Cell 85, 403-414

158. DiSepio, D., M. Sutter, A.T. Johnson, R.A. Chandraratna and S. Nagpal (1999) Identification of the AP1-antagonism domain of retinoic acid receptors [In Process Citation]. Mol Cell Biol Res Commun 1, 7-13

159. Wadgaonkar, R., K.M. Phelps, Z. Haque, A.J. Williams, E.S. Silverman and T. Collins (1999) CREB-binding protein is a nuclear integrator of nuclear factor-kappaB and p53 signaling. J Biol Chem 274, 1879-1882

160. Webster, G.A. and N.D. Perkins (1999) Transcriptional cross talk between NF-kappaB and p53. Mol Cell Biol 19, 3485-3495

161. Petrij, F., R.H. Giles, H.G. Dauwerse, J.J. Saris, R.C.M. Hennekam, M. Masuno, N. Tommerup, G.-J.B. van Ommen, R.H. Goodman, D.J.M. Peters and B. M.H. (1995) Rubenstein-Taybi syndrome caused by mutations in the transcriptional co-activator CBP. Nature 376, 348-351

162. Tanaka, Y., I. Naruse, T. Maekawa, H. Masuya, T. Shiroishi and S. Ishii (1997) Abnormal skeletal patterning in embryos lacking a single Cbp allele: a partial similarity with Rubinstein-Taybi syndrome. Proc Natl Acad Sci U S A 94, 10215-20

163. Kawasaki, H., R. Eckner, T.-P. Yao, K. Taira, R. Chiu, D.M. Livingston and K.K. Yokoyama (1998) Distinct roles of the co-activators p300 and CBP in reinoic-acid-induced F9-cell differentiation. Nature 393, 284-289

164. Dignam, J.D., R.M. Lebovitz and R.G. Roeder (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res 11, 1475-1489

165. Kamakaka, R.T., M. Bulger and J.T. Kadonaga (1993) Potentiation of RNA polymerase II transcription by Gal4-VP16 during but not after DNA replication and chromatin assembly. Genes Dev 7, 1779-1795

166. Gerritsen, M.E., A.J. Williams, A.S. Neish, S. Moore, Y. Shi and T. Collins (1997) CREB-binding protein/p300 are transcriptional coactivators of p65. Proc Natl Acad Sci U S A 94, 2927-2932

167. Gu, W., X.L. Shi and R.G. Roeder (1997) Synergistic activation of transcription by CBP and p53. Nature 387, 819-823

168. Lee, J.S., R.H. See, T. Deng and Y. Shi (1996) Adenovirus E1A downregulates cJun- and JunB-mediated transcription by targeting their coactivator p300. Mol Cell Biol 16, 4312-4326

169. Lill, N.L., S.R. Grossman, D. Ginsberg, J. DeCaprio and D.M. Livingston (1997) Binding and modulation of p53 by p300/CBP coactivators. Nature 387, 823-827

170. Puri, P.L., M.L. Avantaggiati, C. Balsano, N. Sang, A. Graessmann, A. Giordano and M. Levrero (1997) p300 is required for MyoD-dependent cell cycle arrest and muscle-specific gene transcription. Embo J 16, 369-383

171. Eckner, R., M.E. Ewen, D. Newsome, M. Gerdes, J.A. DeCapiro, J.B. Lawrence and D.M. Livingston (1994) Molecular cloning and functional analysis of the adenovirus E1A-associated 300-kD protein (p300) reveals a protein with properties of a transcriptional adaptor. Genes Dev. 8, 869-884

172. Kwok, R.P.S., J.R. Lundblad, J.C. Chrivia, J.P. Richards, H.P. Bächinger, R.G. Brennan, S.G.E. Roberts, M.R. Green and R.H. Goodman (1994) Nuclear protein CBP is a coactivator for the transcription factor CREB. Nature 370, 223-226

173. Swope, D.L., C.L. Mueller and J.C. Chrivia (1996) Identification and characterization of multiple transcriptional activation domains in the CREB binding protein. J. Biol. Chem. 271, 28138-28145

174. Nakajima, T., C. Uchida, S.F. Anderson, C.G. Lee, J. Hurwitz, J.D. Parvin and M. Montminy (1997) RNA helicase A mediates association of CBP with RNA polymerase II. Cell 90, 1107-1112

175. Yang, X., V.V. Ogryzko, J. Nishikawa, B. Howard and Y. Nakatani (1996) A p300/CBP-associated factor that conpetes with the adenoviral E1A oncoprotein. Nature 382, 319-324

176. Yuan, Z.M., Y. Huang, T. Ishiko, S. Nakada, T. Utsugisawa, H. Shioya, Y. Utsugisawa, Y. Shi, R. Weichselbaum and D. Kufe (1999) Function for p300 and not CBP in the apoptotic response to DNA damage. Oncogene 18, 5714-5717

177. Keech, C.A., S.M. Jackson, S.K. Siddiqui, K.W. Ocran and A. Gutierrez-Hartmann (1992) Cyclic adenosine 3',5'-monophosphate activation of the rat prolactin promoter is restricted to the pituitary-specific cell type. Mol. Endocrinol. 6, 2059-2070

178. Peers, B., P. Monget, M.A. Nalda, M.L. Voz, M. Berwaer, A. Belayew and J.A. Martial (1991) Transcriptional induction of the human prolactin gene by cAMP requires two cis-acting elements and at least the pituitary-specific factor Pit-1. J. Biol. Chem. 266, 18127-18134

179. Yan, G.-z., W.T. Pan and C. Bancroft (1991) Thyrotropin-releasing hormone action is mediated by the POU protein Pit-1. Mol. Endocrinol. 5, 535-541

180. Hoggard, N., J.R. Davis, M. Berwaer, P. Monget, B. Peers, A. Belayew and J.A. Martial (1991) Pit-1 binding sequences permit calcium regulation of human prolactin gene expression. Mol Endocrinol 5, 1748-1754

181. Kapiloff, M.S., Y. Farkash, M. Wegner and M.G. Rosenfeld (1991) Variable effects of phosphorylation of Pit-1 dictated by the DNA response elements. Science 253, 786-789

182. Howard, P.W. and R.A. Maurer (2000) Identification of a conserved protein that interacts with specific LIM homeodomain transcription factors. J Biol Chem 275, 13336-13342

183. Fischberg, D.J., X. Chen and C. Bancroft (1994) A Pit-1 phosphorylation mutant can mediate both basal and induced prolactin and growth hormone promoter activity. Mol. Endocrinol. 8, 1566-1573

184. Okimura, Y., P.W. Howard and R.A. Maurer (1994) Pit-1 binding sites mediate transcriptional responses to cyclic adenosine 3',5'-monophosphate through a mechanism that does not require inducible phosphorylation of Pit-1. Mol. Endocrinol. 8, 1559-1565

185. Howard, P.W. and R.A. Maurer (1994) Thyrotropin releasing hormone stimulates transient phosphorylation of the tissue-specific factor, Pit-1. J. Biol. Chem. 268, 28662-28669

186. Maurer, R.A. (1985) Selective binding of the estradiol receptor to a region at least one kilobase upstream from the rat prolactin gene. DNA 4, 1-9

187. d'Emden, M.C., Y. Okimura and R.A. Maurer (1992) Analysis of functional cooperativity between individual transcription-stimulating elements in the proximal region of the rat prolactin gene. Mol. Endocrinol. 6, 581-588

188. Sun, P., H. Enslen, P.S. Myung and R.A. Maurer (1994) Differential activation of CREB by Ca^{2+}/calmodulin-dependent protein kinases type II and type IV involves phosphorylation of a site that negatively regulates activity. Genes Dev. 8, 2527-2539

189. Roberson, M.S., A. Misra-Press, M.E. Laurance, P.J.S. Stork and R.A. Maurer (1995) A role for mitogen-activated protein kinase in mediating activation of the glycoprotein hormone α-subunit gonadotropin-releasing hormone. Mol. Cell. Biol. 15, 3531-3539

190. MacGregor, G.R. and C.T. Caskey (1989) Construction of plasmids that express E. coli β-galactosidase in mammalian cells. Nuc. Acids Res. 17, 2365

191. Crews, C.M., A. Allessandri and R.L. Erikson (1992) The primary structure of MEK, a protein kinase that phosphorylates the *ERK* gene product. Science 258, 478-480

192. Jacob, K.K., J. Sap and F.M. Stanley (1998) Receptor-like protein-tyrosine phosphatase α specifically inhibits insulin-increased prolactin gene expression. J. Biol. Chem. 273, 4800-4809

193. Alessi, D.R., A. Cuenda, P. Cohen, D.T. Dudley and A.R. Saltiel (1995) PD 098059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase *in vitro* and *in vivo*. J. Biol. Chem. 270, 27489-27494

194. Marais, R., J. Wynne and R. Treisman (1993) The SRF accessory protein Elk-1 contains a growth factor-regulated transcriptional activation domain. Cell 73, 381-383

195. Jacob, K.K. and F.M. Stanley (1994) The insulin and cAMP response elements of the prolactin gene are overlapping sequences. J. Biol. Chem. 269, 25515-25520

196. Yang, S.-H., P.R. Yates, A.J. Whitmarsh, R.J. Davis and A.D. Sharrocks (1998) The Elk-1 ETS-domain transcription factor contains a mitogen-activated protein kinase targeting motif. Mol. Cell. Biol. 18, 710-720

197. Zanger, K., L.E. Cohen, K. hashimoto, S. Radovick and F.E. Wondisford (1999) A novel mechanism for cyclic adenosine 3',5'-monophosphate regulation of gene expression by CREB-binding protein. Mol. Endocrinol. 13, 268-275

198. Meier, B.C., J.R. Price, G.E. Parker, J.L. Bridwell and S.J. Rhodes (1999) Characterization of the porcine Lhx3/LIM-3/P-Lim LIM homeodomain transcription factor. Mol. Cell. Endocrinol. 147, 65-74

199. Day, R.N., J. Liu, V. Sundmark, M. Kawecki, D. Berry and H.P. Herlsholtz (1998) Selective inhibiton of prolactin gene transcription by the ETS-2 repressor factor. J. Biol. Chem. 273, 31909-31915

200. Sgouras, D.N., M.A. Athanasiou, G.J. Beal, Jr., R.J. Fisher, D.G. Blair and G.J. Mavrothalassitis (1995) ERF: an ETS domain protein with strong transcriptional repressor activity, can suppress *ets*-associated tumorigenesis and is regulated by phosphorylation during cell cycle and mitogenic stimulation. EMBO J. 14, 4781-4793

201. Bryant, L.A., P. Mixon, M. Davidson, A.J. Bannister, T. Kouzarides, J.H. Sinclair (2000) The human cytomegalovirus 86-kilodalton major immediate-early protein interacts physically and functionally with histone acetyltransferase P/CAF. J. Virol. 16, 7230-7237.

202. McMahon C, T. Suthiphongchai, J. DiRenzo, M.E. Ewen (1999) P/CAF associates with cyclin D1 and potentiates its activation of the estrogen receptor. Proc. Natl. Acad. Sci. USA 10, 5382-5387

203. Korzus E., J. Torchia, D.W. Rose, L. Xu, R. Kurokawa, E.M. McInerney, T.M. Mullen, C.K. Glass, M.G. Rosenfeld. (1998) Transcription factor-specific requirements for coactivators and their acetyltransferase functions. Science 279, 703-707

204. Janknecht, R., A. Nordheim. (1996) MAP kinase-dependent transcriptional coactivation by Elk-1 and its cofactor CBP. Biochem. Biophys. Res. Commun. 228, 831-837

205. Ait-Si-Ali, S., D. Carlisi, S. Ramirez, L.C. Upegui-Gonzalez, A. Duquet, P. Robin, B. Rudkin, A. Harel-Bellan, D. Trouche. (1999) Phosphorylation by p44 MAP Kinase/ERK1 stimulates CBP histone acetyl transferase activity in vitro. Biochem. Biophys. Res. Commun. 262, 157-162